Particle size and source; effects on allergy adjuvant activity and innate immunity

by

Mari Samuelsen

Dissertation submitted for the degree of Philosophiae Doctor

Institute of General Practice and Community Medicine

Faculty of Medicine

University of Oslo

Department of Environmental Immunology

Division of Environmental Medicine

Norwegian Institute of Public Health

2008

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 696

ISBN 978-82-8072-296-6

Cover: Inger Sandved Anfinsen.
Printed in Norway: AiT e-dit AS, Oslo, 2008.

Produced in co-operation with Unipub AS.
The thesis is produced by Unipub AS merely in connection with the
thesis defence. Kindly direct all inquiries regarding the thesis to the copyright
holder or the unit which grants the doctorate.

Unipub AS is owned by
The University Foundation for Student Life (SiO)

Acknowledgements

The work presented in this thesis was carried out at the Norwegian Institute of Public Health, Division of Environmental Medicine during the period of January 2001 to June 2008. Financial support was provided by the Norwegian Academy of Science and Letters and Statoil (VISTA) from 2001-2005 (Grant no. 6141) and the Norwegian Institute of Public Health from 2005-2008, which I most greatly acknowledge.

I wish to thank Erik Dybing, former Head of the Division of Environmental Medicine, for providing an excellent working environment and for being my contact person at the Faculty of Medicine, University of Oslo. I also wish to thank Per Nafstad for taking over as my contact person in 2005.

I want to thank all my colleagues for their invaluable help and support during my period as a PhD student. Firstly, I would like to thank my main supervisor, Professor Martinus Løvik, for all his discussions and guidance during the experimental work, the writing of the papers on which this thesis is based, and finally for providing the essential keywords that made lines connect. I am also thankful that he never ceased to believe in me or this project. Further, I am extremely grateful to my supervisor, Dr. Unni Cecilie Nygaard, for her fruitful discussions, rapid and constructive feedback on manuscript drafts, all the encouraging comments and "smilies", and for always keeping her door open.

Furthermore, I want to thank:

- Else-Carin Groeng, Bodil Hasseltvedt, Åse Eikeset, Astri Grestad and Berit A. Stensby for invaluable help with the animals and laboratory work,
- Trude Olsen and her colleagues at the Research Animal Unit for providing excellent technical assistance,
- Anette Kocbach for interesting and fruitful discussions in the field of particle toxicology, and for all her professional, as well as personal support,
- Dr. Torunn Alberg for her helpful discussions on adjuvant effects of particles and, together with Dr. Jitka Stilund Hansen, the critical reading of the manuscripts,
- Dr. Ellen Namork for proof-reading of this thesis,
- Britt Rydjord, Linda Kathrine Ellertsen and Randi Jacobsen for all their useful discussions on immunology, but most of all for being good friends; supportive, warm and whose company was both stimulating and enjoyable,

- PhD students, Postdocs and all other colleagues at MIMI for warmly including me in their group and who provided a nice and unique working atmosphere.

Finally, I want to thank both my parents and my parents-in-law, for making it possible to combine family life with the position as a PhD student by helping with the children. A special thanks to my husband, Morten Hegge, who has patiently supported me throughout these years, who for longer periods of time has endured the life of a “lonely father” and who has put up with the capricious temper of a wife struggling to finish her thesis. And thanks to my lovely children, Herman and Hennie, who were born during this period, for distracting me with their laughter and chaos, and for always reminding me of what is really important in life.

Oslo, September 2008
Mari Samuelsen

Sammendrag

De siste tiårene har det vært en kraftig økning i antall tilfeller av astma og allergi. Økt partikkelforurensning fra veitrafikk har blitt assosiert med både forverring og utvikling av astma og allergi. Til tross for at vedfyring mange steder er en viktig kilde til partikkelforurensning i uteluft, vet vi lite om hva slags konsekvenser vedfyringspartikler har for folks helse. På bakgrunn av dette ønsket vi å undersøke om partikler fra vedfyringsrøyk kan bidra til økt allergiutvikling på samme måte som partikler fra veitrafikk.

I dette doktorgradsprosjektet fant vi at partikler fra vedfyringsrøyk økte utviklingen av allergi hos mus på lik linje med blandede veitrafikkpartikler bestående av eksospartikler og veistøv. Effekten av vedfyringspartiklene var imidlertid noe lavere enn for eksospartikler alene. I tillegg hadde partikler fra veitrafikk samlet utenfor piggdekksesongen, med høyt innhold av små forbrenningspartikler, større forsterkereffekt på allergiutvikingen enn partikler samlet i løpet av piggdekksesongen, hvor andelen store mineralpartikler var dominerende. Også når store og små modellpartikler ble sammenlignet, viste det seg at de små partiklene hadde størst forsterkereffekt på allergiutviklingen. Når store og små partikler ble gitt i luftveiene til mus for å se på akuttreaksjon, ga de største partiklene (på størrelse med piggdekkstøv) og de minste partiklene (på størrelse med eksospartikler) ulik type reaksjon fra slimhinnen. Dette kan tyde på at eksospartikler og piggdekkstøv påvirker luftveiene forskjellig og kan gi forskjellig type helseskade.

En reduksjon i utslippene av de minste partiklene fra både vedfyring og biltrafikk vil kunne være et viktig bidrag for å redusere forekomsten av allergi og andre luftveisplager.

Contents

Abbrevations

AM	alveolar macrophage
APC	antigen presenting cell
CBP	carbon black particles
COPD	chronic obstructive pulmonary disease
DC	dendritic cell
DEP	diesel exhaust particles
IL	interleukins
MCP-1	monocyte chemoattractant protein 1
MIP-2	macrophage inflammatory protein-2
MHC	major histocompatibility complex
NF-κB	nuclear factor kappa B
NK	natural killer cell
OVA	ovalbumin
PAH	polyaromatic hydrocarbons
PLN	popliteal lymph node
PM	particulate matter
PSP	polystyrene particles
ROS	reactive oxygen species
Th1	T helper 1 lymphocytes
Th2	T helper 2 lymphocytes
TLR	toll-like receptor
TNF-α	tumour necrosis factor α

1. List of papers

This thesis is based on the following publications that will be referred to in the text by their respective Roman numerals:

I

Samuelsen M, Nygaard UC and Løvik M. Allergy adjuvant effect of particles from wood smoke and road traffic. *Toxicology* 2008; 246: 124-131.

II

Nygaard UC, Samuelsen M, Aase A and Løvik M. The capacity of particles to increase allergic sensitisation is predicted by particle number and surface area, not by particle mass. *Toxicol. Sci.* 2004; 82: 515-524.

III

Samuelsen, M., Nygaard, U. C. and Løvik, M. Particle size determines activation of the innate immune system in the lung. [*submitted*]

IV

Samuelsen, M., Nygaard, U. C. and Løvik, M. Particles from wood smoke and road traffic differently activate the innate immune system of the lung. [*submitted*]

2. Introduction

2.1. Particulate air pollution and human health

Extreme episodes of air pollution, like the Meuse valley fog in Belgum in 1930 and the even more famous "London smog" in 1952, were associated with thousands of excess deaths. Since then, effective legislation has led to the elimination of most of the air pollution of 50 years ago, like sulphur dioxide and black smoke from the burning of coal. Nevertheless, the relation between urban air pollution and adverse health effects persists (Brunekreef and Holgate, 2002). One reason may be that new elements have been introduced. Urban air pollution is today dominated by fine combustion particles, nitrogen oxides and ozone, as combustion from vehicles powered by gasoline and diesel is the most important source (Oberdorster and Utell, 2002). A vast amount of epidemiologic studies link exposure to ambient particulate matter (PM) with respiratory and cardiovascular morbidity and mortality (Pope and Dockery, 2006), and the annual number of premature deaths due to particle exposure has been estimated to be 800.000 worldwide (WHO, 2002). A range of cardiopulmonary diseases have been associated with exposure to particles with diameters smaller than 10 μm, including lung cancer, atherosclerosis and reduced lung development in children, as well as the exacerbation of airway diseases like allergy, asthma, and COPD (Alfaro-Moreno *et al.*, 2007; Brook *et al.*, 2004; Heinrich and Wichmann, 2004; Kappos *et al.*, 2004).

In industrialised countries, the prevalence of allergic airway diseases has increased dramatically over the last decades (Beasley *et al.*, 2000; Schafer and Ring, 1997). This rapid increase is unlikely to be caused by genetic changes in the population. Various environmental risk factors linked to what is called a "western lifestyle" have been associated with this increase in prevalence such as changes in diet (Devereux, 2006), reduced infections in childhood (Strachan, l989), less exposure to endotoxin or other bacterial products (Braun-Fahrlander, 2003), altered intestinal microflora (Bjorksten *et al.*, 1999), excessive skin washing with loss of barrier function (Callard and Harper, 2007), and environmental changes in indoor (Anderson and Bogdan, 2007) and outdoor air quality (Heinrich and Wichmann, 2004; Saxon and Diaz-Sanchez, 2005). Traffic-related air pollution is one factor that has gained much attention, and has indeed been associated with the exacerbation of asthma and allergic diseases (Heinrich and Wichmann, 2004; Saxon and Diaz-Sanchez, 2005), and even

the development of such diseases (Annesi-Maesano *et al.*, 2007; Brauer *et al.*, 2007; Janssen *et al.*, 2003; Morgenstern *et al.*, 2008). Diesel exhaust particles (DEP), which is a major constituent of combustion particles in urban areas (Ho *et al.*, 2006; Zheng *et al.*, 2002), have both in humans and in animal models been shown to enhance allergic responses to allergens, as reviewed by Riedl and Diaz-Sanchez (2005).

Although some studies suggest that exposure to particles generated from the burning of wood may lead to adverse respiratory health effects (Boman *et al.*, 2003; Lipsett *et al.*, 1997; Naeher *et al.*, 2007; Orozco-Levi *et al.*, 2006; Schreuder *et al.*, 2006), the effect of wood smoke particles have been much less studied. This is so despite the fact that wood smoke particles are a primary source of combustion particles in several countries, at levels comparable to the contribution from vehicle exhaust (Glasius *et al.*, 2006; Wu *et al.*, 2007). Additionally, wood smoke particles and DEP have several characteristics in common, such as particle size and associated metals and PAHs (Kocbach *et al.*, 2006), and are thus likely to exert adverse health effects similar to DEP. Therefore, further investigations on wood smoke particles in relation to adverse health effects is needed, including studies on how these particles affect the development of allergic responses. Furthermore, more work has to be done to elucidate which particle characteristics are involved in the particle adjuvant effects on allergic sensitisation.

Despite our knowledge that air pollutants interact with both the innate and the adaptive immune system to alter immunophysiologic outcomes (section 2.4), further information is warranted concerning the mechanisms that underlie these outcomes, and which particle characteristics are involved. It is also important to find how these airborne particles affect the mucosa-associated innate immune system so that allergic sensitisation against co-administered antigen is increased.

2.1.1. Definitions

Particulate matter (PM) is classified according to the aerodynamic diameter of the particles, which is defined as the geometrical diameter of a smooth spherical particle that has a density of 1 g/cm^3 and the same settling speed in still air as the particle in question (Phalen, 2002). Ambient air particles are thus divided into three classes according to size; coarse (2.5 to 10 μm in diameter), fine (0.1 to 2.5 μm in diameter), and ultrafine particles (<0.1 μm in diameter) (Phalen, 2002). These particles are often referred to as inhalable particles. Previously, particle concentrations in air were monitored as mass concentration of PM_{10}

(particles with an aerodynamic diameter less than 10 µm), as PM_{10} is assumed to be the upper size limit of particles affecting the lungs. However, an increasing number of studies have indicated that the level of $PM_{2.5}$, and recently also $PM_{0.1}$, is more closely related to adverse health effects and mortality than PM_{10} (Penttinen *et al.*, 2001; Peters *et al.*, 1997; Pope and Dockery, 2006; von Klot *et al.*, 2002). Therefore, mass concentrations of $PM_{2.5}$ are also frequently being monitored. Since ultrafine particles, in spite of high numbers, constitute very little of the total particle mass in ambient air (Figure 1), monitoring of this particle fraction would require measurement of number concentrations.

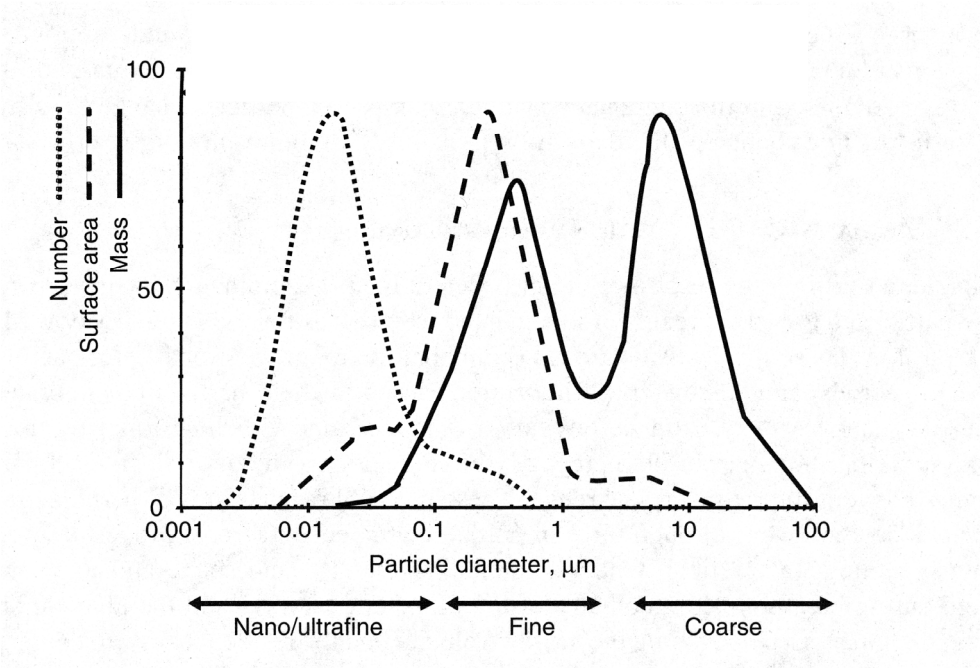

Figure 1. Typical particle size distributions in urban air with regard to number, mass and surface area (from Kreyling et al., 2007)

2.1.2. Particle sources and composition

PM in ambient air is a complex mixture of multiple components ranging from a few nanometers in size to tens of micrometers. Particles in the atmosphere may be classified as primary, referring to particles emitted directly from sources, or secondary, referring to

particles formed within the atmosphere from gas-to-particle conversion processes. Primary particles are directly emitted as solid particles or liquid droplets from either natural or anthropogenic sources like forest fires, soil erosion, sea salts, incomplete burning of fossil fuels, road dust, biomass burning, or from different types of industry including waste-disposal plants (Kreyling *et al.*, 2007).

PM emitted from the various sources is highly diverse in physical and chemical properties such as size, surface area, morphology, crystal structure, surface charge and chemical composition. The coarse particle fraction predominantly consists of inorganic minerals, like windblown dust from crustal erosion, while fine and ultrafine particles are mainly derived from the combustion of fossil fuels. During the cold season, however, the amounts of wood smoke particles may reach high levels locally (Glasius *et al.*, 2006; Wu *et al.*, 2007). Combustion particles both from traffic and wood smoke are mainly carbon aggregates that consist of spherical primary carbon particles with diameters ranging from 20 to 50 nm (Kocbach *et al.*, 2005; Kocbach *et al.*, 2006; Paoletti *et al.*, 2002). The emitted ultrafine primary particles grow by coagulation and surface deposition in ambient air to form chains and clusters of carbon core particles in the fine size fraction (Lighty *et al.*, 2000). The small diameters of the primary particles provide a large surface area per mass, which allows for adsorption of various compounds like metals and PAHs (BeruBe *et al.*, 2007) as well as biological material like endotoxin and allergens (Harrison and Yin, 2000; Namork *et al.*, 2006; Ormstad *et al.*, 1998). The larger mineral particles, however, exhibit a smaller surface area per mass compared to the carbon aggregates and thus adsorb less chemical substances per mass unit. Ambient air particles may be subjected to further alternations by photochemical processes resulting in sulphur coating and modification of organic compounds (Paoletti *et al.*, 2002; Vione *et al.*, 2006).

2.1.3. Particle deposition and clearance

The main function of the respiratory system is gas exchange through the uptake of oxygen and the excretion of carbon dioxide that occurs across the epithelial cells lining the alveoli. However, inhaled air contains a wide range of particles and it has been shown that residence in a region with high levels of ambient particles results in pulmonary retention of large quantities of fine and ultrafine particle aggregates (Brauer *et al.*, 2001).

The dose of inhaled particulate matter is a function of both deposition on airway surfaces as well as clearance from those surfaces. Each of these variables may be modulated

by factors such as age, gender, pre-existing disease and physical activity (Kreyling *et al.*, 2007). The mechanisms of deposition are, in large part, determined by the physiochemical characteristics of the particles, like size, shape, density and charge. Large particles are more susceptible to the anatomical air filtration, and will deposit in the upper airways and in the major conducting airways, while the smaller particles are able to reach deeper into the airway system (Figure 2; Phalen, 2002). However, the deposition is also affected by biological breathing patterns (volume and rate), route of breathing (mouth vs. nose), and the anatomy of the airways (Bennett and Brown, 2005).

Once deposited on the epithelial surface, the morphology of chain-aggregated particles may change, either by compaction or disintegration depending on particle surface and epithelial lining fluid properties (Gehr *et al.*, 2000). Because of this liquid layer, the associations between the particle core and attached substances also will change depending on water and lipid solubility (Kreyling *et al.*, 2007).

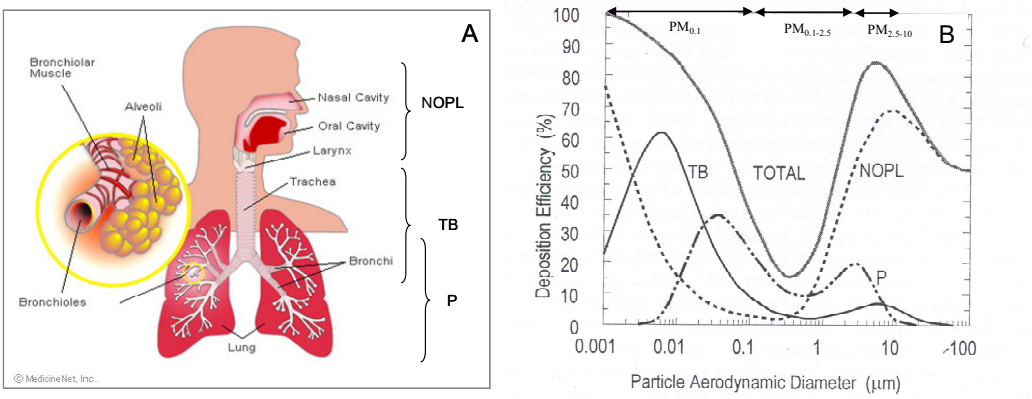

Figure 2. A) A schematic illustration of the human respiratory tract, divided into the nasopharyngeal region (nose, mouth and throat), the tracheobronchial region (TB), and the pulmonary or alveolar region (P). Modified from http:/www.associateprogramsales.com /asthma/index3.html. B) Particle deposition in the major regions of the human respiratory tract during normal respiration, corrected for size dependent inhalability (Phalen, 2002).

Particles deposited in conducting airways settle at the mucus layer which is transported by beating cilia towards the larynx (Figure 3), from where they are swallowed into the gastrointestinal tract. The absence of mucociliary action in the peripheral lung (alveoli) results in much slower particle clearance. Insoluble particles deposited in the alveolar region will be taken up by the alveolar macrophages (AMs) within a few hours after deposition, and transported slowly towards the ciliated airways inside AMs (Kreyling *et al.*, 2007). Particles engulfed by dendritic cells (DCs) located at the base of the epithelium (Figure 3), on the other hand, are transported into the tissue from where they reach the lymph nodes via the lymphatic system (Byersdorfer and Chaplin, 2001). Additionally, particles may be bound to or taken up by the epithelial lining cells, and thus be retained in the lungs for a longer period of time (Kreyling *et al.*, 2007).

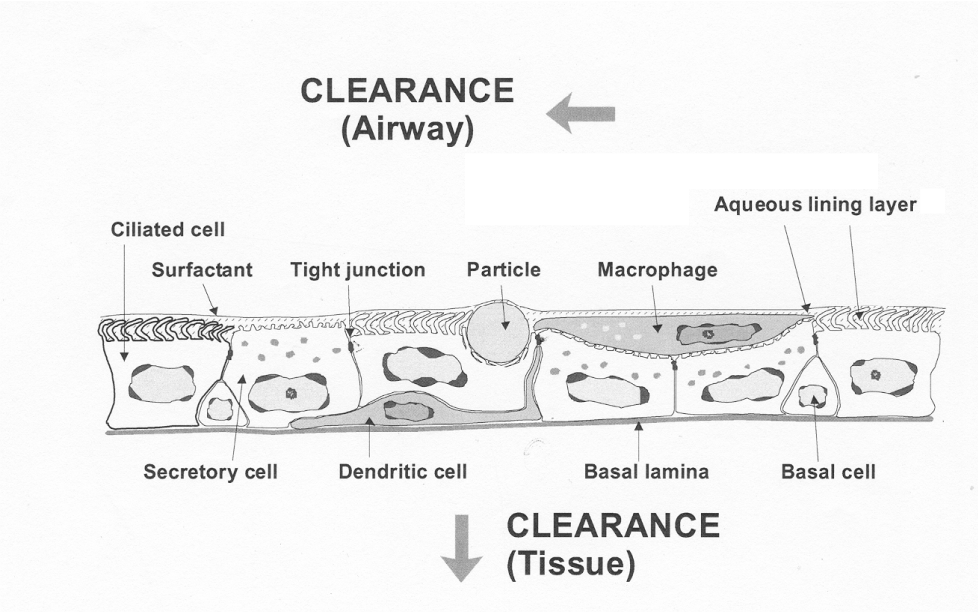

Figure 3. Schematic drawing of airway epithelial barrier with macrophages and dendritic cells, exposed to a fine particle. Modified from McWilliam et al. (2000).

Macrophage-mediated particle removal may be impaired, especially in elderly, smokers, and subjects with lung diseases (Moller *et al.*, 2001). Moreover, macrophages are less able to take up ultrafine particles, even in healthy lungs (Kreyling *et al.*, 2006). Ultrafine particles may also be cleared by translocation across the epithelial cell lining into the blood vessels towards secondary target organs like heart and brain (Kreyling *et al.*, 2006; Nemmar *et al.*, 2002).

2.2. The innate immune system of the lung

The respiratory epithelial cell surface presents a large fragile interface with the external environment, and is continuously exposed to a diverse array of airborne particles and invading microorganisms during respiration. In order to protect the host against such challenges, a complex defence system normally involving both innate (non-specific) and acquired (specific) immune responses, has evolved. The pulmonary immune system is tightly regulated to avoid excess inflammation and fibrosis, that may compromise the main function of the lung, namely gas exchange. The innate immune system is the front line of host defence, always ready to recognise and respond to different types of "danger signals". Coughing and sneezing as well as the mucociliary blanket remove most of the larger particulates from the upper airways, as described in section 2.1.3. In the deeper parts of the lung, however, the innate immune system is very well developed. Innate defence is made up of a humoral arm (defensins, surfactant proteins, mannose binding lectin etc) and a cellular arm, consisting mainly of resident and recruited phagocytic cells.

2.2.1. Cells involved in the early innate immune response

The air space of the naive lung is mainly populated by AMs. These scavenger cells continuously ingest dead or dying cells as well as particles and environmental pathogens without inducing inflammation or activation of the adaptive immunity, to avoid airway damage to harmless antigens (Lambrecht, 2006; MacLean *et al.*, 1996). AMs express several types of cell surface receptors; scavenger receptor MARCO (macrophage receptor with collagenous structure) and Scavenger receptor A (SR-A), lectins, integrins, Fcγ-receptors and complement receptors involved in the phagocytosis of foreign material (Underhill and Ozinsky, 2002). Several of these receptors are also expressed on other phagocytic cells such as monocytes, neutrophils and DCs (Underhill and Ozinsky, 2002). While phagocytosis via

Fcγ-receptors leads directly to inflammation, in many cases the decisions to activate inflammatory responses during phagocytosis are regulated by additional receptors that are not themselves phagocytic (Ozinsky *et al.*, 2000). Toll-like receptors (TLRs), members of the family of pattern-recognition receptors, are one example. TLRs recognise distinct conserved structural components of pathogens (PAMPS; pathogen-associated molecular patterns), and evoke inflammatory responses upon binding (Akira, 2003). The killing of pathogens requires phagocytosis along with activation signals leading to the generation of reactive oxygen and nitrogen species essential in microbial defence (Fang, 2004). As one of the major effector cells of the innate immune system, activated macrophages play a crucial role in controlling and directing immune responses in the lung by secreting a variety of soluble mediators.

In normal, healthy lungs, very few neutrophils can be observed in the alveolar space (Cohen and Rossi, 1983). However, in the presence of lung infection and inflammation, neutrophils are recruited into alveolar spaces to reinforce the airways host defence. Within 3 to 4 h after challenge by an infectious agent, neutrophils may constitute 60% to 80% of the total cells recovered by bronchoalveolar lavage (BAL) (Zhang *et al.*, 2000). The recruited neutrophils become functionally activated via stimulation by pro-inflammatory cytokines and other mediators released within the infected compartment. Neutrophils have high phagocytic activity and are important in the killing of microbes (Segal, 2005). Monocytes are also recruited to the site of inflammation, and usually appear inside the alveoli within 24 to 48 h (Larsen and Holt, 2000). Freshly recruited monocytes display a pro-inflammatory phenotype with high phagocytic activity, that after a few days are differentiated to macrophages in the alveolar environment (Bilyk and Holt, 1995).

The airway epithelial cells have also been proven to play an important role in innate host defence (Mayer and Dalpke, 2007). Beside their function as barriers, epithelial cells produce several anti-microbial substances as well as inflammatory mediators, many of which are initiated by pathogen-recognition receptors such as TLR on the epithelial surface (Mayer and Dalpke, 2007; Wang *et al.*, 2008). Below the epithelial lining of the mucosa resides a tight network of another type of phagocytic cells, the dendritic cells (DCs, section 2.3.3.). These cells pick up any invading substances and bring them to secondary lymphoid organs of the airway system (Banchereau and Steinman, 1998). Different subsets of DCs are involved in the induction of tolerance to inhaled antigens, as well as classical priming of the adaptive immune effector cells called T lymphocytes described in section 2.3.2. (Hammad and Lambrecht, 2007).

2.2.2. Pro-inflammatory mediators

The initiation, maintenance, and resolution of pulmonary innate responses depend upon cellular communication via cytokines. Along with other soluble factors, as well as adhesion molecules, the cytokines contribute to the recognition of pathogens on the cell population level, the recruitment of neutrophils and mononuclear cells, and the removal of the invading agent.

Cytokine signalling occurs through receptor-ligand interactions on specific immune or non-immune target cell populations. Pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and IL-8 (macrophage inflammatory protein (MIP)-2 in mice) may initiate and exacerbate inflammation, whereas the anti-inflammatory cytokines IL-10, IL-4, and IL-13 serve to reduce and regulate the inflammatory response and promote healing (Dinarello, 2000; Guo and Ward, 2002; Murphy *et al.*, 2008).

Two of the most important early-response cytokines in innate immunity are TNF-α and IL-1β. Macrophages are regarded as the major source of TNF-α (Driscoll, 2000), while IL-1β is in addition produced by epithelial cells (Murphy *et al.*, 2008). These two cytokines initiate the expression and release of a cascade of pro-inflammatory cytokines, including TNF-α and IL-1β themselves, but also other cytokines like IL-6, IL-8, MIP-2 and granulocyte macrophage-colony stimulating factor (GM-CSF), resulting in the recruitment and activation of other cells (Dinarello, 2000; Driscoll *et al.*, 1997; Driscoll, 2000). In mice, the two most important chemokines for neutrophil recruitment into the lung are MIP-2 and keratinocyte-derived chemokine (KC) which are produced by epithelial cells, endothelial cells, AMs and fibroblasts (Driscoll, 2000; Reutershan and Ley, 2004). The chemotactic factor monocyte chemoattractant protein 1 (MCP-1), mainly produced by the same cells (Van Coillie *et al.*, 1999), is a member of the CC-family of chemokines which play an important role in the recruitment of monocytes to the inflammatory site (Strieter *et al.*, 2002). The complement activation product C5a, like MIP-2 and KC, plays an important role in increasing the influx of neutrophils (Guo and Ward, 2002).

2.2.3. Linking the innate and adaptive immune system

Innate immune responses are increasingly recognized as critical modifiers of adaptive immunity (Arredouani *et al.*, 2007; Cook and Bottomly, 2007). The dependency on innate

immune cells for adaptive immunity to develop is mainly caused by the need for antigen presentation, a function carried out by APCs, and DCs in particular (Banchereau and Steinman, 1998). Under baseline conditions, AMs produce different mediators including nitrogen oxide, IL-10 and transforming growth factor (TGF) β, that directly suppress the induction of adaptive immunity through their effects on DCs and T cells (Lambrecht, 2006; Lipscomb *et al.*, 1993). This balance has been shown to shift upon stimulation of surface receptors on macrophages, such as TLR (Takabayshi *et al.*, 2006). The activation of AMs may affect the activation status of DCs both through relief of suppression, but also as a consequence of cytokines released from these macrophages (Nicod *et al.*, 2000). Epithelial cells have also been shown to play an important role in the recruitment and activation of DCs by secreting chemokines and cytokines in response to "danger signals" (Hammad and Lambrecht, 2008).

Allergic airway inflammation develops in the context of innate immune cells which express TLRs that seem to be important in linking the innate and adaptive immune system. TLRs have been identified not only on macrophages, but on other APCs, neutrophils, epithelial cells, fibroblasts and mast cells, as well as specific types of T cells (Akira *et al.*, 2006; Applequist *et al.*, 2002). Stimulation of TLRs has been suggested to be involved in activation and maturation of DCs, differentiation of various T cell subsets, activation of airway epithelial cells, cytokine production in mast cells, and activation of eosinophils (Iwamura and Nakayama, 2008).

Although it is clear that TLRs expressed on APCs are essential in the initiation of an adaptive response, interaction between APCs also with other parts of the innate immune system such as natural killer (NK) cells and factors of the complement system, may trigger the maturation of APCs and thus activate adaptive immunity (Hoebe *et al.*, 2004).

2.3. Allergy

Adaptive immune responses are elicited by inherently harmless "environmental" antigens such as pollen, food, and drugs, and this may unfortunately, lead to harmful immune reactions known generally as hypersensitivity reactions. Hypersensitivity reactions have previously been classified into four types by Coombs and Gell (Murphy *et al.*, 2008). Allergy, the most common type of hypersensitivity, has traditionally been classified as type I (IgE-mediated) and type IV (cell mediated) hypersensitivity reactions. Throughout this thesis the

word allergy refers to the IgE-mediated reactions only. To describe IgE-mediated disease, the term “atopy” is often used. Individuals with atopy have a genetic predisposition to produce IgE antibodies against common environmental allergens and have one or more atopic diseases (Kay, 2001). Why some antigens, called allergens, in some individuals cause an allergic response whereas they in other individuals are harmless, is still unknown, although several susceptibility genes have been discovered (Vercelli, 2008). It appears that protein allergens can be grouped into a limited number of families, which suggest that they have certain properties in common (Radauer *et al.*, 2008).

2.3.1. Effector mechanisms

The allergic reaction results from two temporally distinct processes, the sensitisation and challenge phases (Figure 4; Holgate and Church, 1993). In the sensitisation phase, allergens enter via the mucosal surface and are taken up by DCs residing in the sub-epithelial tissue. The DCs process and present the allergen in major histocompatibility complex (MHC) class II molecules to naïve T lymphocytes in the draining lymph node. If antigen specific T cells recognise the allergen (epitopes) through their T cell receptor (TCR), and the proper co-stimulatory factors are present, the T cells are stimulated to proliferation and differentiation, and become capable of stimulating B lymphocytes to produce antigen-specific IgE antibodies (section 2.3.4). The IgE molecules bind to mast cells via high affinity Fcε receptors (FcεRI) and become sensitised (Holgate and Church, 1993; Murphy *et al.*, 2008). During the challenge phase, when allergens subsequently reaches the sensitised mast cell, the allergens cross-link surface bound IgE, causing the immediate release of both pre-formed and newly synthesised mediators such as histamine, proteases, leukotrienes and prostaglandins. IgE-mediated degranulation of mast cells and the resulting inflammation may produce clinical symptoms of allergic disease (Gould and Sutton, 2008). The early phase of the immediate allergic reaction can be followed after 6-8 h by a more sustained inflammation, known as the late-phase response. As a result of cytokine or mediator release the site of inflammation becomes infiltrated with effector cells such as T helper (Th) 2 cells (see next section), eosinophils, basophils, neutrophils, mononuclear cells, and mast cells further sustaining the inflammatory reaction (Gelfand, 2004).

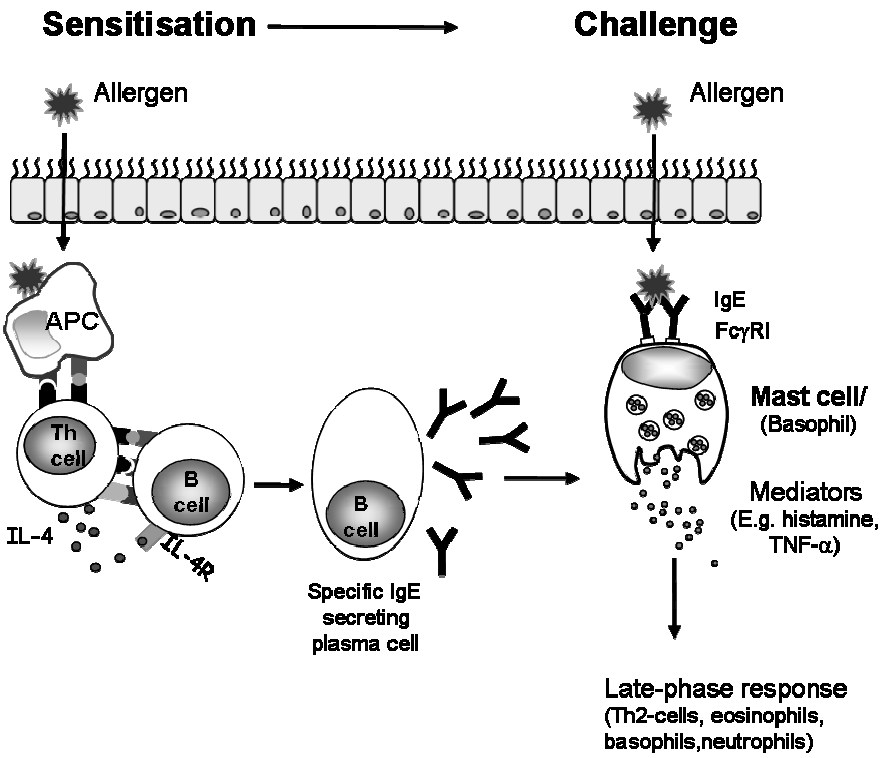

Figure 4. Schematic illustration of immunologic mechanisms involved in development of an allergic response. Modified from Holgate and Church (1993).

2.3.2. T lymphocyte subsets

Based on their phenotype, two main T lymphocyte subsets can be distinguished: T cytotoxic cells (Tc) which express CD8 molecules on their surface, and T helper cells which express CD4 molecules. Naive $CD8^+$ T cells recognise antigen presented by MHC class I present on all nucleated cells in the body, and differentiate into cytotoxic effector T cells that recognise and kill cells displaying "abnormal" antigens. Naive $CD4^+$ T cells, on the other hand, have a more flexible repertoire of potential effector activities (Murphy *et al.*, 2008; Oboki *et al.*, 2008). After recognition of antigen presented by MHC class II molecules on APCs, naïve $CD4^+$ T cells can differentiate into different effector subsets with different immunological functions.

Traditionally, two subsets of Th cells have been described based on their cytokine secreting profile, namely Th1 and Th2 cells. Th1 cells typically secrete IFN-γ and IL-2, whereas Th2 cells typically secrete IL-4, IL-13 and IL-5 (Mosmann *et al.*, 1986; Romagnani, 2001). Microbes, particularly intracellular microbes, often skew the response towards a Th1 type, which promote "cellular" immune responses characterised by activation of cytotoxic T cells and macrophages, and production of opsonising IgG2a (rodent; IgG1 in humans) antibodies. On the other hand, the Th2 cells play an important role in "humoral" immune responses (antibody responses), characterised by production of IgE and IgG1 (rodent; IgG4 in humans), sensitisation of mast cells, and eosinophil cell recruitment and maturation. The biological function of a Th2 response is typically to mount an adaptive response towards helminthic worms more commonly found in less developed countries. The same responses are unfortunately also activated by allergens in susceptible individuals and are an important cause of disease in the industrialised countries (Kay, 2001; Schafer and Ring, 1997).

2.3.3. Factors affecting Th2 differentiation

Although many different factors influence the differentiation of Th cells into Th1 and Th2 cell subsets, the cytokine environment is believed to be of great importance. IFN-γ and IL-12 are thought to be the major cytokines for promoting Th1 differentiation, whereas high levels of IL-4 and low levels of IL-12 drives the precursor Th cells towards a Th2 differentiation (Lambrecht, 2001; Mosmann *et al.*, 1986). IFN-γ is produced by natural killer (NK) cells and cytotoxic T cells, while IL-12 is produced by macrophages and dendritic cells. The source of "early IL-4" is not clear, however both basophils, eosinophils, mast cells, and natural killer T (NKT) cells have been suggested (Akbari *et al.*, 2003; Haas *et al.*, 1999). However, the Th1/Th2 paradigm has proven to be overly simplistic (Kidd, 2003). An immune response is usually a mixed Th1/Th2 response (Gor *et al.*, 2003; Kidd, 2003), and the presence of IFN-γ has even been suggested to enhance allergic inflammation (Rowe *et al.*, 2004). Moreover, in addition to the traditional Th1 and Th2 effector subsets, Th17 has been identified as a novel Th effector cell (Infante-Duarte *et al.*, 2000; Oboki *et al.*, 2008). Adding to the complexity of immune regulation, several regulatory T cell subsets with inhibitory activity that limits the extent of immune activation and inflammation, have been identified (Larche, 2007).

DCs are primary antigen-presenting cells involved in the interactions with T cells leading to the differentiation and proliferation of Th cell subsets. Upon antigen uptake, DCs migrate to draining lymph nodes, where they present the antigen to naive T cells (Banchereau and Steinman, 1998). The immunologic consequences of antigen presentation in draining lymph nodes depend largely on the DC's maturation state, determined in part by the inflammatory conditions within the airways. Under steady-state conditions antigen presentation generally invokes T cell unresponsiveness and thus tolerance to the antigen (Hawiger *et al.*, 2001). Some factors favouring Th2 polarisation by DCs are absence or low levels of secreted IL-12, the presence of certain TLR agonists, or proteolytic activity within the allergen (Eisenbarth *et al.*, 2003; Hammad and Lambrecht, 2008). However, the hypothesis that Th2 differentiation occurs by default in the absence of Th1-induced stimuli has also been launched (Eisenbarth *et al.*, 2003). Finally, the development of a Th2-skewed environment during an immune response have been shown to be determined by genetic background (Vercelli, 2008), the presence of environmental factors (adjuvants; Granum and Lovik, 2002), and route, dose and frequency of allergen exposure (Nelde *et al.*, 2001).

2.3.4. Regulation of IgE isotype switch in B lymphocytes

After exposure to an antigen, the first antibodies to be produced by allergen-specific B cells are IgM and IgD. In order to become IgE-producing plasma cells the mature (naive) B cells need a number of molecular signals provided by the Th2 cells. Th2 cells can produce the cytokines necessary for stimulation of B cells to IgE class switch, namely IL-4 and IL-13 (Finkelman *et al.*, 1990). Vercelli and co-workers demonstrated that physical contact between Th and B cells is mandatory for the isotype switch to occur (Vercelli *et al.*, 1989). Allergen associated with MHC class II molecules on the B cell surface is presented to Th2 cells and recognised by the T cell receptor (TCR) complex (Figure 5). This interaction leads to the secretion of IL-4, as well as the expression of the CD40 ligand (CD40L) on the T cell surface, leading to the two distinct signals required for B cells to undergo isotype switching (Armitage *et al.*, 1993; Jabara *et al.*, 1990). The first signal involves the binding of IL-4 to its receptor (IL-4R) on B cells, and results in transcription of the Cε heavy chain gene locus which does not code for the full-length ε heavy chain. Further, the CD40L transiently expressed on the T cell surface interacts with CD40 on the B cell surface, thus delivering the second signal which triggers deletional switch recombination leading to transcription of the whole ε heavy chain

(Bacharier and Geha, 2000). The ligation of CD40L and CD40 (as well as the ligation of IL-4R and IL-4) also results in up-regulation of several B cell surface molecules, including CD80/86 which serves as receptor for CD28 expressed on T cells. The interaction of CD80/86 with CD28 results in optimal Th2 cell activation and increased secretion of cytokines, including IL-4 (reviewed in Poulsen, 2000).

Until recently, IgE class switch has been thought to occur only in the germinal centres of lymphoid tissue, whereas the production of IgE mainly originated from memory plasma cells recruited from the bone marrow to the circulation. However, recent findings demonstrate that both IgE class switch and IgE production may occur locally in the respiratory tract mucosa in individuals with allergic disease (Gould and Sutton, 2008; KleinJan *et al.*, 2000).

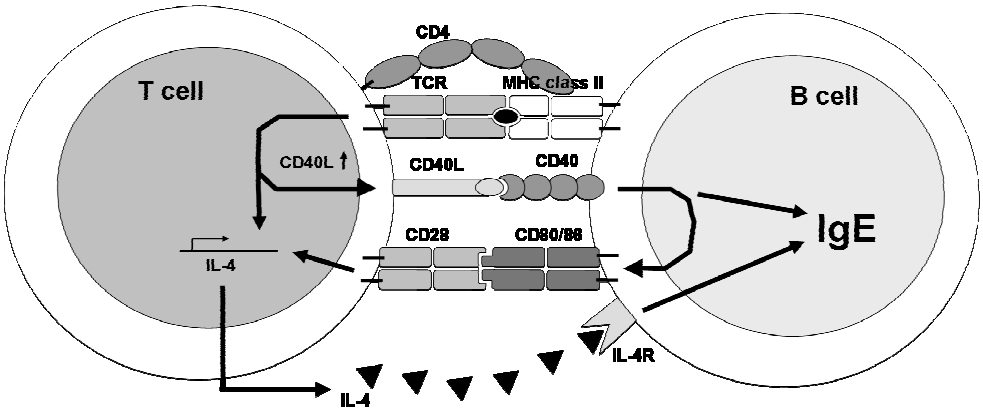

Figure 5. Proposed sequence in activation of B cells to become IgE producers (modified from Bacharier and Geha, 2000).

2.4. The effect of particles on innate and allergic immune responses

Humans are not "allergic" to common air pollutants; that is, people do not generate adaptive immune responses to pollutants *per se*. The important issue is how airborne pollution interacts with mucosal surfaces and associated immune tissues to modulate the immune

responses leading to adverse health outcomes. It has been suggested that most airborne pollutants function as mucosal adjuvants and, in interacting with both innate and adaptive immune cells, skew the immune response to inhaled antigens toward a Th2 phenotype (Parnia *et al.*, 2002; Saxon and Diaz-Sanchez, 2005).

2.4.1. DEP, a model air pollution particle

Widely different types of particles have been shown to enhance allergic responses to allergen in animal models (Dybing *et al.*, 2004; Granum and Lovik, 2002). Particularly DEP have been subjected to intense research, and their capacity to induce nasal and pulmonary inflammation, as well as their adjuvant capacity regarding development and intensity of allergic immune responses, has been demonstrated both in humans and in animal models (Riedl and Diaz-Sanchez, 2005). DEP have been shown to trigger inflammatory responses through interaction with innate immune cells such as the airway epithelial cells and macrophages, resulting in the release of pro-inflammatory cytokines and chemokines from both cell types (Devalia *et al.*, 1997; Takizawa, 2004). *In vitro* exposure to diesel-enriched particles have been suggested to lead to DC maturation via the activation of epithelial cells, favouring the activation of Th2 cells (Bleck *et al.*, 2006; Bleck *et al.*, 2008). Increased recruitment of inflammatory cells such as neutrophils, macrophages and lymphocytes, as well as increased levels of inflammatory cytokines and expression of adhesion molecules have been observed in BAL fluid and nasal washes of healthy people exposed to diesel exhaust (Behndig *et al.*, 2006; Diaz-Sanchez *et al.*, 2000a; Salvi *et al.*, 1999). These findings are supported by numerous studies in animals using intratracheal instillation or inhalation of DEP (Nel *et al.*, 1998; Rao *et al.*, 2005; Saber *et al.*, 2005; Saber *et al.*, 2006).

After exposure of rodents to DEP and the allergen ovalbumin (OVA) eosinophil infiltration, enhanced airway hyperresponsiveness and histamine release have been observed, suggesting that DEP are able to turn a minimal mucosal response to allergen challenge into a robust one (Miyabara *et al.*, 1998; Nel *et al.*, 1998; Takano *et al.*, 1997). Moreover, DEP in combination with OVA have previously been shown to induce allergic sensitization against the allergen (Lovik *et al.*, 1997; van Zijverden *et al.*, 2000), suggesting that DEP may have the potential to enhance the development of allergic responses. In support of these findings, DEP have been shown to both induce (with allergen) and exacerbate *in vivo* allergic responses in the human upper respiratory tract, as observed in human nasal provocation models (Diaz-Sanchez *et al.*, 1997; Diaz-Sanchez *et al.*, 1999). The deviation into a Th2 milieu has been

reported, observed as increased allergen-specific IgE production accompanied by increased expression of IL-4, IL-5 and IL-13. Moreover, DEP-induced *in vivo* isotype switching to IgE in allergic humans have been reported (Fujieda *et al.*, 1998). Finally, it has also been suggested that DEP can enhance the severity of clinical symptoms to the allergen by enhancing mast cell and basophil degranulation and cytokine release both *in vivo* and *in vitro* (Devouassoux *et al.*, 2002; Diaz-Sanchez *et al.*, 2000b).

2.4.2. Mechanisms of particle effects

To explain the effects of particle exposure, several mechanisms have been suggested. DEP and ambient air particles have been reported to interact with innate immune receptors like TLRs, and may possibly mediate their effects through direct triggering of such receptors (Becker *et al.*, 2005; Inoue *et al.*, 2006). On the other hand, it has been reported that the small size of ultrafine particles may enable access to intracellular organelles by passive diffusion through cell walls. Thus particles may also mediate their effects through direct interaction with organelles such as mitochondria (Peters *et al.*, 2006; Xia *et al.*, 2006). Moreover, particles have been suggested to function as a carrier of allergen, leading to increased deposition of allergen in the lower parts of the lung, as further discussed in section 6.1.3. In addition, the uptake, processing and presentation of the allergens in the APC might be affected by the presence of particles (Parnia *et al.*, 2002). Exposure to particulate matter may also cause the disruption of epithelial barriers, resulting in easier access of allergen to APCs and other cells of the immune system (Murphy *et al.*, 1998). However, the importance of inflammation and generation of reactive oxygen species (ROS) in mediating particle induced health effects have recently received increased attention (Frampton, 2006; Li *et al.*, 2008). Based mainly on *in vitro* studies it has been suggested that the effects of DEP and other particles may be driven by increased cellular oxidative stress causing the activation of MAP kinase and NF-κB transcription factors which regulate gene expression of cytokines, chemokines and adhesion molecules involved in inflammation (Li *et al.*, 2003; Xiao *et al.*, 2003). Furthermore, generation of oxidative stress has been suggested to induce activation of APCs and increase CD80 and CD86 expression on their surface, enhancing their ability to interact with Th cells and to skew the response in a Th2 direction (Becker and Soukup, 2003; Don Porto *et al.*, 2002; Hamilton *et al.*, 2004; Nel *et al.*, 1998).

2.4.3. The importance of particle characteristics

Particles often have inorganic and organic materials attached to their surface. Transition metals and chemical components, as well as bacterial products such as endotoxin and β-glucans, have been shown to induce inflammation and increased allergic sensitisation (Gerhold *et al.*, 2002; Instanes *et al.*, 2004; Lambert *et al.*, 2000; Ma and Ma, 2002; Schwarze *et al.*, 2007). Metals and PAHs have also been shown to induce the generation of ROS (Ghio *et al.*, 2002; Ma and Ma, 2002). However, low-chemical or chemical free particles such as carbon black particles (CBP), titanium dioxide particles (TiO_2) and polystyrene particles (PSP) have proven to have similar biological effects in rodents, suggesting that the particle core play an important role in inducing inflammatory responses (Brown *et al.*, 2001; Donaldson *et al.*, 2000; Stoeger *et al.*, 2006), as well as in increasing the IgE response towards the allergen (Granum *et al.*, 2001a; Granum *et al.*, 2001b; Lovik *et al.*, 1997; Nygaard *et al.*, 2005a; van Zijverden *et al.*, 2001).

The focus on the importance of particle size and particle surface area in relation to human health has increased (BeruBe *et al.*, 2007; Donaldson *et al.*, 2005), and some epidemiological studies have indicated that ultrafine particles are mainly responsible for the reported respiratory effects of particulate air pollution (Ibald-Mulli *et al.*, 2002; Penttinen *et al.*, 2001; Peters *et al.*, 1997). Importantly, these particles have a higher deposition rate, poorer clearance and greater potential to cross epithelial barriers. Ultrafine particles may thus reach immune cells in the interstitial space in the lung and even be transported to secondary organs via the blood stream (see section 2.1.3.). These particles have been reported to cause damage by inducing inflammation and oxidative stress because of their large biologically active surface to which toxic chemicals might adhere (Donaldson *et al.*, 2001; Peters *et al.*, 2006).

3. Aims of the study

The main aim of this study was to investigate the adjuvant effect of particles from wood smoke and road traffic on the development of allergic sensitisation. Combustion engine derived exhaust particles have been shown to affect the immune system and the respiratory mucosa. Besides vehicle exhaust, wood smoke is a primary source of combustion particles in several countries. The biological effect of wood smoke particles has been less investigated. We therefore wanted to compare particles from wood smoke with traffic related particles with regard to allergy adjuvant activity. Further, we wanted to compare the two types of particles with regard to activation of the innate immune system, with the expectation that this might give information relevant for the understanding of differences between the particles also in relation to allergy.

We aimed to answer the following questions:

1. What is the capacity of wood smoke particles to enhance allergic sensitisation compared to particles from road traffic? What is the contribution of coarse mineral particles and ultrafine combustion particles to the adjuvant effect of road traffic particles? (Paper I).
2. How does particle size influence the adjuvant effect of particles on allergic sensitisation? (Paper II)
3. Is particle size important also for other aspects of innate immune system function than antigen presentation and co-stimulatory activity, like innate immune cell activation? (Paper III)
4. Do particles from wood smoke and road traffic induce cellular activation differently? (Paper IV)

4. Summary of the papers

Paper I

Allergy adjuvant effect of particles from wood smoke and road traffic

The purpose of this study was to compare the adjuvant effect of particles from wood smoke and road traffic on allergic sensitisation, measured as serum allergen-specific IgE levels, cell membrane markers and *ex vivo* cytokine production. Wood smoke particles, mixed road traffic particles, carbon black particles (CBP) and DEP with and without OVA were injected subcutaneously into the footpad of BALB/cA mice, followed by an OVA booster injection three weeks later. Wood smoke particles and mixed road traffic particles increased the IgE response to OVA to similar levels, while DEP had the greatest effect, followed by CBP. We also compared the enhancing effects of road traffic particles collected during winter (St+; studded tires) and autumn season (St-; no studded tires) on OVA-specific IgE production. The St- sample, which had the highest content of small combustion particles, increased the IgE response to OVA significantly more than St+.

The adjuvant effects of particles from wood smoke, road traffic and DEP on the cellular response in the popliteal lymph node (PLN) were studied five days after a single injection into the footpad of particles with or without OVA. All particles tested with OVA increased the PLN cell numbers and cell proliferation, as well as the expression of various cell surface molecules (CD19, MHC class II, CD86 and CD23), and *ex vivo* secretion of the cytokines IL-4 and IL-10. DEP showed the greatest adjuvant effect also on cellular responses, followed by wood smoke particles, while mixed road traffic particles had the lowest adjuvant effect.

Over all, wood smoke and road traffic particles had about the same capacity to enhance allergic sensitisation, but less than DEP. Also, the amount of small combustion particles seemed to be an important factor concerning the adjuvant capacity of road traffic particles.

Paper II

The capacity of particles to increase allergic sensitisation is predicted by particle number and surface area, not by particle mass

In this study, the effect of particle size on allergic sensitisation was explored, using the same footpad immunisation model as described in paper I. Clean, chemical-free PSP ranging in size from coarse to ultrafine (diameters 11.14, 4.646, 1.053, 0.202 and 0.064 μm), CBP and DEP with and without OVA, were injected into one hind footpad of mice. Fine and ultrafine

particles (0.0588 and 0.202 μm PSP, DEP and CBP) increased the production of OVA-specific IgE to considerably higher levels than the larger particles (1.053, 4.64 and 11.14 μm PSP). Furthermore, the PLN cell numbers, expression of cell surface molecules (CD19, MHC class II, CD86 and CD23) and ex vivo cytokine production of IL-4 and IL-10 increased with decreasing particle diameter after injection of the same mass concentration of 0.202, 1.053 and 11.14 μm PSP with OVA.

In conclusion, particle size seem to be an important factor when addressing the adjuvant effect of particles on allergic sensitisation, smaller particles having the greatest biological effect.

Paper III

Particle size determines activation of the innate immune system in the lung

In this study, we wanted to investigate whether particle-induced cellular activation within the innate immune system also is dependent on particle size. Chemical-free polystyrene particles (PSP) ranging from coarse to ultrafine (4.646, 1.053, 0.202 and 0.064 μm) were instilled intratracheally into BALB/cA mice. Simultaneously, one day or seven days after particle exposure, mice were inoculated with the intracellular bacterium *Listeria monocytogenes* and bacterial numbers in the lungs were determined one day after bacterial challenge. In separate experiments, BAL fluid was collected 4 and 24 h after particle instillation, about the time of bacterial challenge. Differential cell counts were performed, and the levels of several pro-inflammatory mediators, as well as markers of cytotoxicity and tissue damage were measured in the BAL fluid.

When mice were simultaneously exposed to PSP and *Listeria*, a reduction in bacterial numbers were observed only in the groups exposed to 0.202 and 0.064 μm PSP, indicating a rapid cell activation induced by the two smallest PSP. When PSP were given one day prior to bacterial challenge, also the largest PSP (4.646 μm) induced reduction of bacterial numbers in the lung, while the fine-sized 1.053 μm PSP still had no detectable effect on this aspect of the innate immune response. Neutrophil numbers, however, were increased in all PSP exposed groups, but tended to be highest in the group exposed to the largest PSP (4.64 μm). Also, significantly higher levels of TNF-α, MIP-2 and IL-1β were measured in BAL fluid of the group exposed to 4.64 μm PSP than in the group exposed to 0.064 μm PSP, which induced no increased levels of pro-inflammatory mediators.

Cellular activation after exposure to chemical-free particles thus was found to be dependent on size, seen as a biphasic pattern (bell-shaped curve) in the reduction of bacterial

numbers. While coarse particles seemed to lead to cellular activation through a TNF-α mediated pathway, other mechanisms e.g. oxidative stress might have been involved after exposure to ultrafine particles.

Paper IV

Particles from wood smoke and road traffic differently activate the innate immune system of the lung

The aim of this study was to compare the effect of particles from wood smoke and road traffic on innate immune system cellular activation. Instillation of wood smoke particles, mixed road traffic particles collected during the winter with use of studded tires (St+) and during the autumn without use of studded tires (St-), and DEP with subsequent *Listeria* challenge was performed as described in paper III. BAL fluid was also collected and analysed as described above.

All particles tested activated the innate immune system, measured as a significant reduction in bacterial numbers after particle exposure. DEP had the most rapid effect, since DEP induced the greatest reduction compared to the other particles when given simultaneously with *Listeria*. When particles were given one day prior to *Listeria*, however, the bacterial numbers in all particle exposed groups were reduced to similar levels. The particle effect was transient, since no reduction in bacterial numbers was observed when particles were instilled 7 days prior to *Listeria* challenge. The observation of a particle induced innate immune response, was supported by an increase in total cell numbers, mainly due to an influx of neutrophils. Exposure to St+, St- and DEP increased the levels of MIP-2, IL-1β and MCP-1 in BAL fluid, while only St+ and St- increased TNF-α levels. The presence of coarse particles may explain the high levels of pro-inflammatory mediators induced by St+ and St-, although the results might also have been influenced by the endotoxin content in these particle samples. In contrast, wood smoke particles did not induce increased levels of any of the pro-inflammatory mediators measured, but some increase in LDH activity, indicative of cytotoxicity, was detected.

Exposure to particles from wood smoke and road traffic induced cellular activation seen as reduction in bacterial numbers, as well as influx of neutrophils. The differences in levels of pro-inflammatory mediators measured in BAL fluid, however, indicated that the innate immune system was activated by different mechanisms.

5. Methodological considerations

5.1. Particles and particle preparations

5.1.1. Particles used in the study

When particles from wood smoke and road traffic were collected, a high-volume sampler was not available. Therefore, to obtain sufficient amounts of particles for biological experiments, road traffic particles were collected in a road tunnel with high traffic load. For sampling, polycarbonate filters connected to a pump through a specially designed manifold was used, as described by Kocbach et al. (2006). Wood smoke particles were collected directly from a conventional wood stove by isokinetic particle sampling on polycarbonate filters (Kocbach *et al.*, 2006).

People exposed to ambient air particles from road traffic are likely to be exposed to a mixture of engine-derived combustion particles, mineral and bitumen particles from road abrasion and wear particles from brakes and tires. The road traffic particles used in the biological experiments were likely to contain all these elements, although these particle samples were dominated by the mass concentration of combustion and mineral particles (Kocbach *et al.*, 2006). Moreover, the combustion particles were generated from a wide range of vehicles, including cars, trucks and busses fuelled with diesel or gasoline. The road traffic particles we used are therefore likely to be relevant for ambient human traffic exposures. In addition to road traffic particles sieved at 10 μm, we used particles collected during two different seasons, resulting in different proportions of combustion particles and mineral particles in the two samples. Particles collected during the winter season when studded tires are used (St+), contained a greater proportion of mineral particles compared to particles collected during the autumn season when no studded tires are used (St-) (Kocbach *et al.*, 2006). This difference in mineral particle content was caused by the use of tires equipped with metal studs to avoid sliding on icy road surfaces in winter time, resulting in high road abrasion.

Wood smoke particles were collected from a conventional wood stove (Kocbach *et al.*, 2006) with similar combustion technology as used in wood stoves and boilers accounting for the majority of wood smoke particle emissions in the Nordic countries (Sternhufvud *et al.*, 2004). However, the physiochemical properties of wood smoke particles vary considerably depending on temperature and air supply (Boman *et al.*, 2003; Kocbach *et al.*, 2005). Ambient

wood smoke particles originate from a wide range of combustion conditions, while the wood smoke particles used in our experiments were collected only under high temperature conditions, and not exposed to atmospheric chemical processes. However, it has been reported that mainly particles with morphology typical of wood smoke particles generated under high temperature are found in ambient air (Kocbach *et al.*, 2005), and therefore the particles we used in this study were considered to be of relevance to human exposure. Additionally, when the *in vitro* inflammatory capacity of the wood smoke particles were compared to particles collected during different phases of the combustion cycle, similar responses were detected (Schwarze *et al.*, 2008).

Diesel exhaust particles were used as a representative of one type of traffic combustion particles without mineral particles. The Standard Reference Material (SRM) 2975 (National Institute of Standards Technology, Gaithersburg, MD, USA) were collected from a diesel-powered industrial forklift, and thus might not be a good representative of contemporary diesel emissions. Moreover, there exists a large variation of DEP emitted from different sources, and one selected particle source can not represent human DEP exposure in general. However, SRM 2975 is well-characterised and therefore a suitable reference diesel exhaust particle for comparison with other particle samples in terms of chemical composition and biologic activity. Carbon black particles (Regal 250) were used as a surrogate for the combustion particle core without associated chemicals.

When addressing the effect of particle size on the immune system, polystyrene particles of different size with no chemicals adsorbed to their surface were used. Polystyrene particles consist of polymerised styrene (CH_2=CHC_6H_5), and exhibit a slight negative charge from sulphate esters (Polyscience Europe GmbH, Eppheim, Germany). The advantages of these particles are the uniform particle sizes (coefficient of variance between 2-5%) and the commercial availability of a range of sizes.

Two different batches of PSP were used in this project as representatives of ultrafine particles. PSP with a diameter of 0.0588 μm were used in the experiments addressing the allergy adjuvant effect of particles (paper II), while 0.064 μm PSP were used when the particle effect on innate immunity was investigated (paper III). The 0.0588 μm PSP formed presumably loose aggregates after dilution with buffer or OVA-solution. Since vehicle and wood smoke combustion particles consist of aggregated primary particles with a diameter of about 30 nm (Kocbach *et al.*, 2005), 0.0588 μm PSP were considered to be a suitable model for the core of aggregated combustion particles (paper IV). However, when diluting 0.0588

μm PSP in buffer to the desired concentration for intratracheal instillation, a suspension of visible, highly agglomerated particles was obtained. Intratracheal instillation of such heterogeneous suspensions would result in a very uneven distribution of particles in the lung, as well as danger of blocking the airways. Therefore, 0.064 μm PSP, which are considerably more homogenous in suspension, were used in these experiments. During the polymerisation process of this batch of particles, sodium dodecyl sulfat (SDS) was used to avoid aggregation. We cannot rule out that traces of this surfactant may be present in these particle preparation (<0.1% present during production; personal communication with manufacturer). However, in "worst case", after diluting the particle samples to desired concentrations for instillation, the content of SDS was less than 0.004% and probably much lower. This concentration was not likely to influence the results, since the effect of 0.03% SDS had no significant effect on bacterial numbers in the lung when compared to control (unpublished results). Furthermore, the 0.202 μm PSP which did not contain SDS, gave similar results as 0.064 μm PSP regarding both reduction in bacterial numbers and parameters measured in BAL fluid.

5.1.2. Particle preparations and agglomeration

In this study, mice were exposed to aqueous solutions of combustion particles and in this regard it is important to consider the issue of particle agglomeration. All our samples containing combustion particles, toghether with the 0.0588 μm PSP, formed agglomerates. However, ultrafine particles in ambient air naturally form agglomerates (Katrinak *et al.*, 1993), and one would assume that a certain degree of agglomeration is relevant to human exposure.

In the footpad immunisation model, particle suspensions were stirred for 18 h prior to injection, while sonication was used before intratracheal instillation. Previously, we have compared different ways of suspending DEP in both the footpad immunisation model and the *Listeria* challenge model. No significant differences in biological response to sonicated versus stirred particle suspensions were found (unpublished data). However, to reduce the possibility of heavily centralised particle deposition in the lung, more homogenous suspensions were obtained by sonication before intratracheal instillation, as recommended by others (Driscoll *et al.*, 2000).

Despite agglomeration of particles in solution, we have chosen to present the primary particles sizes. This parameter can be used to range the particles according to their surface area, which strongly influence the surface area of the agglomerates. Surface area calculations

based on TEM measurements for particles larger than 20 nm correlate well with BET-based specific surface area (Wittmaack, 2007), and this parameter is used as an important factor in explaining the biological effects of particles (Paper II). Moreover, it is difficult to present meaningful, "real" particle sizes, because the size varies from single particles to large, relatively loosely formed agglomerates (1-5 μm) as observed in TEM seen in the case of DEP. We do not know the fate of these agglomerates once they are injected into the footpad or instilled into the lungs of mice, and we do not know the biological effects of agglomerates compared to solutions of single particles. We have observed that the OVA-specific IgE response after injection with homogenous suspensions of 0.064 μm PSP seemed to be somewhat lower than after exposure to the more agglomerated 0.0588 μm PSP, indicating that a certain degree of agglomeration might even enhance the adjuvant effect of particles (unpublished results).

5.1.3. Particle doses

In the studies of the cellular responses after injection of particles and allergen into the footpad, three different particle doses were used (100 μg in paper I, versus 40 and 200 μg in paper II). The 100 μg dose used in paper I was chosen based on a similar study by Nygaard et al. (2005) and was, in accordance with the present findings, sufficient to obtain differences between groups exposed to different particles.

Dose-response studies were performed to compare bacterial numbers after instillation of different doses of DEP one day prior to *Listeria* (100,000 bacteria; unpublished data). The lowest concentration of DEP that resulted in significant, reproducible differences in bacterial numbers between DEP exposed mice and control animals was chosen, namely 100 μg (1 mg/ml).

The particle doses were within the range of probable real-life doses, as it has been claimed that 100 μg of DEP is inhaled during 1-3 days in Los Angeles (Saxon and Diaz-Sanchez, 2000). However, to discuss the relevance of doses makes no sense when it comes to the footpad injection model, which differs strongly from the real-life inhalation situations. In real life, the dose is distributed over a large mucosal surface, whereas with injection exposure in particular, but also with intratracheal instillation, the dose is concentrated to a much smaller area, and the dose expressed e.g. as amount of particles per draining lymph node, will be much higher. Therefore, the exposure models we have used are models suitable only to compare the effects of different particle types and to study the mechanisms behind these

effects, as discussed in section 5.2.1. and 5.3.1. In mechanistic studies and for hazard identification, the particle dose and the exposure route is not as crucial as for risk assessment type of studies (Phalen, 2002).

5.1.4. Endotoxin measurements

The higher endotoxin content in the St+ fraction compared to the St- fraction is in accordance with previous findings, suggesting that endotoxin is associated with the coarse particle fraction (Heinrich *et al.*, 2003). There are, however, uncertainties attached to the endotoxin measurements, and absolute values may not be reliable. The endotoxin levels presented in paper I and IV were measured in the aqueous extract of suspended particles and revealed four times the content in the St+ compared to the St- sample. Cell cultures exposed to these particles in combination with polymyxin B, on the other hand, indicated that there were no differences in the effect of endotoxin between the two samples (Kocbach et al., manuscript submitted).

5.2. The adjuvant immunisation model

To investigate the effect of particles on allergic sensitisation we chose to use BALB/cA mice, regarded as an IgE high-responder strain to OVA (Holt *et al.*, 1981) and therefore considered to be a model for atopic individuals. IgE is the hallmark of allergic disease, a key molecule in the mechanism of allergic reactions, and a useful parameter in models of allergic sensitisation. Female mice were used because they respond more strongly to OVA compared to male mice (Melgert *et al.*, 2005). Additionally, female mice tend to be less aggressive and are thus easier to house and handle. We do not know, if the observed IgE levels would cause allergic symptoms upon challenge. Recent studies, however, have illustrated that the IgE adjuvant effect of particles in an intranasal exposure model was associated with increased numbers of eosinophils in BAL fluid (Steerenberg *et al.*, 2004b). These findings suggest that increased levels of IgE may predict clinical effects.

5.2.1. Footpad immunisation and lymph node cell studies

The subcutaneous injection in footpad immunisation may resemble the situation in the lung after particles have passed the pulmonary mucosa, and are transported to the draining

lymph node within cells (Byersdorfer and Chaplin, 2001; Harmsen *et al.*, 1985). The initial, specific immune response to foreign material that enters the body takes place in the lymph node draining the portal of entry (Zinkernagel, 2000). Importantly, the footpad of mice is drained exclusively to the popliteal lymph node (PLN) (Closs, 1975; Cuq, 1966) and the PLN, therefore, will be the exclusive site of primary immune responses to material injected into the footpad. This makes the footpad-PLN model particularly well suited for cellular response studies (McLachlan *et al.*, 2008). Another advantage of this model over airway exposure is less variation in the retained dose, leading to less experimental variations.

The immune mechanisms found in the footpad model are likely to be involved also in airway exposure models. However, particle effects on the cells in the first line of defence of the airway mucosa are expected to modulate the subsequent immune response (Hammad and Lambrecht, 2008; Iwamura and Nakayama, 2008; Lambrecht, 2006). The influence of local innate immune cell activation and inflammation after footpad injection has not been studied, and might be of less importance compared to airway exposure models. However, our lab has recently shown, in an intranasal exposure model, that the capacity of PSP to increase the IgE response to OVA increases with decreasing particle size (Alberg, unpublished results). These findings are in accordance with the response observed in the footpad injection model in paper II, and suggest that there might be similarities also in innate immune cell activation (in particular dendritic cells) and inflammation leading to similar allergic responses.

The cellular responses in the lymph node could not predict the antibody response, emphasising the importance of the allergen-specific IgE as the primary parameter and that PLN data should be used as supplementary information on cellular mechanisms (Lovik *et al.*, 2007). Discrepancies may be explained by the primary local lymph node response partly being a measure of inflammation and short-term toxic effects and only partly a measure of the specific immune response with dendritic cell- T lymphocyte interactions in focus. Both the primary irritants and immune responses not leading to allergy may contribute to the observed responses, as discussed by Løvik et al. (2007).

5.2.2. Ex vivo cytokine production

When performing the studies of *ex vivo* cytokine secretion by lymph node cells, we used the mitogen Con A as an unspecific stimulating agent to “develop” the cytokine secretion pattern. Even though ConA is widely used in such studies, little has been published on how ConA affects the production of different cytokines in various cell systems. We considered

ConA to be a useful alternative, since we also wanted information on the general particle-effect. While OVA-stimulation for several days would probably have shown the cytokine production from the OVA-specific cells (and bystandards), the shorter ConA-stimulation show the ability of the PLN cells in general to produce cytokines. Since ConA has been used for all particles, a shift in the response induced by ConA would probably be similar for all particles, although artefacts can not be completely ruled out. The observed differences in cytokine production by cells from animals exposed to all the test particles are therefore regarded as useful when comparing particle effects.

5.3. The model of innate immune cell activation

In the studies of particle induced activation of innate immunity, BALB/cA mice were also used. We found it appropriate to use the same strain of mice as in the studies of the IgE adjuvant effect of particles, to better be able to compare the results and thereby make the overall conclusions more valid. The inflammatory responses in mice are less studied, but have been shown to be qualitatively and quantitatively different from those seen in rats, possibly due to species differences concerning lung overload (Bermudez *et al.*, 2004; Elder *et al.*, 2005). More severe inflammation seem to develop in the lungs of rats upon particle exposure, and less relevant responses such as development of lung tumours, have therefore been observed after pulmonary exposure of rats to particulate matter (Heinrich *et al.*, 1995). A disadvantage of mice as test animals was that only small amounts of BAL fluid and lung lavage cells were available for further analysis, thereby strictly limiting the number of parameters that could be measured using available methods.

5.3.1. Intratracheal instillation

Intratracheal instillation is considered a useful tool in hazard screening, to compare particle effects, and to investigate mechanistic differences between particles (Driscoll *et al.*, 2000), which is in accordance with the aims of our study.

Although inhalation is the real life exposure route, intratracheal exposure has some advantages over inhalation since the method requires no special equipment and is often less time-consuming. Another advantage by intratracheal instillation is that an accurate dose of the test material is delivered to the lung in a controlled manner and therefore requires less test material than inhalation methods. Intratracheal instillation may result in a fairly even

distribution of particles in the lung after instillation of an appropriate sample volume (Baxter and Port, 1974; Costa *et al.*, 1986). A volume of 100 µl was instilled in our experiments, to ensure an appropriate distribution of particles and *Listeria*. Instillation of a bacterial inoculum of 100 µl (4 ml/kg) seemed to give a more reproducible number of bacteria one day after inoculation compared to 50 µl, possibly due to less centralised deposition of bacteria (unpublished observations). However, vehicle effects may occur when the instilled volume exceeds 2 ml/kg, and this could possibly have masked some of the particle effects compared to control.

Importantly, the intratracheal route of exposure differs from inhalation concerning dose rate and size-specific particle distribution patterns throughout the airways (Driscoll *et al.*, 2000; Osier and Oberdorster, 1997), factors that probably have affected the observed response. Moreover, the use of particles for instillation lacks the gas phase and its potential effects. It is, however, important to note that intratracheal instillation allowed the study of particles ranging in size from coarse to ultrafine. It has previously been shown that larger particles may be respirable in humans (mouth breathing) but not in rodents, making the study in a rodent inhalation model difficult (Menache *et al.*, 1995; Raabe, 1988).

5.3.2. *Listeria* as an indicator of phagocytic cell activation

In this bacteria lung challenge model, the intracellular bacterium *Listeria monocytogenes* was used as a probe to detect the acute effect of particles on cellular activation during the innate immune response. The lung is not the natural organ for *Listeria* colonisation, but since intracellular pulmonary bacteria like *mycobacterium tuberculosis* are aggressive pathogens which require rigid safety precautions and also long-time experiments because of slow growth, a lung exposure model using *Listeria* has previously been established (van Loveren *et al.*, 1988).

To ensure that only the innate immune function was involved in the studies of particle effects, the experiments were terminated one day after *Listeria* challenge (Lovik and North, 1985; North, 1973). As opposed to our use of this model, *Listeria* has most often been employed to study combined components of the innate and adaptive immunity three to seven days after bacterial inoculation in rats, focusing on the overall resistance to infection after particle exposure (Antonini *et al.*, 2000b; Steerenberg *et al.*, 2004a; Yang *et al.*, 2001; Yin *et al.*, 2002). It is important to note that the acute particle effect on cellular activation, seen as reduction in bacterial numbers in our studies, is not comparable to the effects observed three

or more days after *Listeria* inoculation, as also discussed in paper III and IV. During the course of infection, the effect of particles may change since more cells are gradually recruited and the response will involve both innate and adaptive immune cells (North, 1973).

In the mammalian host, *Listeria* can enter and live in non-phagocytic as well as phagocytic cells. When *Listeria* is phagocytised by non-activated phagocytes, these bacteria escape the phagosomes and thrive in the cytosolic cell environment (Campbell, 1994). However, *Listeria* is highly sensitive to cellular activation (Steinmuller *et al.*, 2000), since the escape of bacteria into the cytosol is prevented in activated phagocytes, and the bacteria are killed inside the vacuoles (Shaughnessy and Swanson, 2007). Activation of innate immunity is therefore important in the first line of defence against *Listeria*. In addition to macrophages and neutrophils (Cousens and Wing, 2000; Shaughnessy and Swanson, 2007), activation of other cells like dendritic cells and mast cells has been suggested to participate in the early defence against *Listeria* (Gekara and Weiss, 2007; Tam and Wick, 2004). Hence, early killing of *Listeria* involves several different cells and *Listeria* may thus be considered a useful indicator of innate immune cell activation in the lung. However, the *Listeria* challenge model we have used provides no information about which types of innate immune cells were activated.

Initial experiments were performed to identify the appropriate number of bacteria for inoculation. Mice were treated with 100 µg DEP one day prior to inoculation with different numbers of *Listeria*. 100,000 *Listeria* was chosen, since a significant difference in bacterial numbers between DEP-treated and control mice, was obtained with this dose. This load of bacteria resulted in a reproducible infection status in the mice during the *Listeria* challenge period of 24 h, and did not make the mice overtly ill.

5.3.3. Parameters measured in BAL fluid

To obtain information on pro-inflammatory and cytotoxic effects after exposure to the different particles, BAL fluid was collected 4 and 24 h after particle instillation, about the time of *Listeria* inoculation. The time point of maximal inflammatory and cytotoxic effects in rats has been claimed to be 24 h after intratracheal instillation, except for some cytokine responses, like TNF-α, which generally peaks at about 4 h (Seagrave *et al.*, 2002). Based on our results in paper III and IV, the time course of the pro-inflammatory responses seemed to be similar in mice.

The parameters chosen for BAL fluid analysis was the pro-inflammatory cytokines TNF-α and IL-1β, the neutrophil and monocyte chemoattractants MIP-2 and MCP-1, as well as LDH and total protein, markers of cytotoxicity and tissue damage with serum leakage from blood, respectively. The number of parameters included in these analyses was restricted by the limited amount of fluid retrieved upon lavage of mice lungs.

5.4. Statistical analysis

When performing the studies, we chose to use a randomised block design to distribute the work load. Hence, the experiments were split into smaller, equal sub-experiments (randomised blocks) carried out at different times (Festing *et al.*, 2002; Ruxton and Colegrave, 2006). All experiments were analysed by analysis of variance (ANOVA). If the ANOVA indicated significant differences, Tukey's post-hoc test was run to determine which groups differed form each other. The parametric two-way ANOVAs require normally distributed data with equal variances. Even though some of the data did not seem to strictly meet the criteria for parametric analysis, the two-way ANOVA was chosen since there were no adequate two-way ANOVAs for non-parametric data available. This approach gave us the advantage to utilise the data from all sub-experiments in one analysis, giving much larger statistical power.

6. General discussion

6.1. Adjuvant effect of particles

In paper I, we found that combustion related particles from different sources enhanced allergic sensitisation. Our results are in agreement with those obtained by others, (Dybing *et al.*, 2004; Granum *et al.*, 2001a; van Zijverden and Granum, 2000), suggesting that particles generally have an adjuvant activity. However, the adjuvant effect of the various particles, measured as the capacity to enhance the specific IgE response to OVA, differed. Wood smoke particles and mixed road traffic particles had similar adjuvant effect, while DEP had the highest effect, followed by CBP.

6.1.1. The effects of particle-associated chemical compounds

As mentioned in section 2.4.3., particle characteristics like size, surface area, associated inorganic and organic substances have been shown to influence allergic sensitisation to co-administered allergen. While the primary particle diameters of the combustion particles in the different samples were comparable, the amount of coarse mineral particles and chemical components associated with the particles varied, as outlined in paper I. Compared to CBP, it seemed like the chemical substances associated with DEP further enhanced the adjuvant capacity of DEP, while the opposite might have been true for wood smoke particles. Possible explanations for the lower adjuvant capacity of wood smoke particles like storage time, content of antioxidants, and toxic compounds have been discussed in paper I. Previously, Barrett and colleagues found that inhalation of hardwood smoke did not cause any significant increase in OVA-specific IgE production in Balb/c mice (Barrett *et al.*, 2006). However, the study lacks a positive control like DEP to verify that the exposure model employed was able to provoke an allergen-specific IgE-response. The fact that allergen-specific IgE was measured early (18 h) after the last allergen challenge, and that the inhalation of particles and allergen was done separately in time may have contributed to this observation.

As reported in papers I and II, CBP induced significantly higher levels of OVA-specific IgG2a than wood smoke and traffic-related particles. This mixed Th1/Th2 response of CBP is supported by previous findings (Lovik *et al.*, 1997; van Zijverden *et al.*, 2000). Among the chemical-free PSPs, only 0.0588 μm PSP + OVA significantly induced specific IgG2a levels.

These findings indicate that the particle core of ultrafine particles without associated chemicals induce a mixed Th1/Th2 response in Balb/cA mice, while particle associated chemicals may drive the response in one direction. The chemicals present on DEP have indeed been shown to skew the response in a Th2 direction (Tsien *et al.*, 1997; Yanagisawa *et al.*, 2006), while the particle core stimulate both Th1 and Th2 responses (Ma and Ma, 2002). Alternatively, it has been suggested that particle associated chemicals might suppress Th1 responses (Ma and Ma, 2002). The latter explanation is in accordance with wood smoke and mixed road traffic particles inducing very low levels of antigen-specific IgG2a, but still significantly lower IgE responses to OVA compared to CBP (paper I).

6.1.2. The effect of particle size

In paper I, we investigated the adjuvant effect of two samples of mixed road traffic particles containing different proportions of combustion and mineral particles. St- particles, which contained the highest proportion of vehicle derived combustion particles, induced significantly higher OVA-specific IgE production than St+ particles. We therefore hypothesised that the combustion particle fraction, consisting of ultrafine primary particles, was mainly responsible for the adjuvant capacity of the mixed road traffic particles. Previous studies have shown that the fine fraction of ambient air particles have a higher adjuvant capacity than the coarse fraction (Dybing *et al.*, 2004; Steerenberg *et al.*, 2005). This supports our observations that the amount of combustion particles is important, since the fine fraction of ambient air particles consists primarily of combustion particles (Paoletti *et al.*, 2002; Shi *et al.*, 2003b). However, St+ and St- were collected during the winter and autumn respectively, and we cannot exclude the possibility that seasonal effects also might have affected the responses as previously observed by others (Steerenberg *et al.*, 2005)

The importance of size in deciding the adjuvant capacity of particles was further investigated in paper II, and we found that the adjuvant activity of chemical-free PSPs increased with decreasing particle diameter at a given mass dose. Differences in adjuvant capacity of particles both within the fine size range and between fine and ultrafine particles are in accordance with the findings of other investigators (de Haar *et al.*, 2005; de Haar *et al.*, 2006; Granum *et al.*, 2000), giving further support to our hypothesis that the combustion particles are particularly important in relation to allergic sensitisation. Hence, the lower adjuvant capacity of road traffic particles compared to DEP at equal weight doses might be

caused by the coarse mineral particles "diluting" the effect of the combustion-generated fraction of the road traffic particles as discussed in paper I.

The observation that ultrafine combustion particles may have adverse health effects is in accordance with reports suggesting an association between cardiopulmonary health effects and the number of ultrafine particles in ambient air (Ibald-Mulli *et al.*, 2002; Kreyling *et al.*, 2006; Penttinen *et al.*, 2001; Peters *et al.*, 1997; von Klot *et al.*, 2002). In general, our data support the growing recognition of particulate air pollution as a contributor to worsening of human health, and that the strategies to reduce the particle emissions should focus on combustion-derived particles which are found in the fine and ultrafine particle fractions.

6.1.3. Particles as allergen carriers

In paper I and II we found that particles with OVA in addition to increasing the allergen-specific IgE response, also increased the primary cellular responses in the lymph node compared to OVA alone. Some of the cellular parameters were weakly altered by OVA alone, and it is therefore possible that particles act by intensifying the inherent immune response towards the allergen, as previously suggested by Nygaard et al. (Nygaard *et al.*, 2005b; Nygaard *et al.*, 2005a). One adjuvant mechanism that could contribute to this is particles acting as allergen carriers.

Many vaccine adjuvants enhance the antibody production by carrying the antigen in multimolecular ("particular") aggregates (Cox and Coulter, 1997). Many types of particles, including combustion generated particles, have been shown to carry allergens on their surface (de Haar, 2006; Namork *et al.*, 2006; Ormstad *et al.*, 1998). The allergen-carrier function of particles possibly make an important contribution to the immune adjuvant potential by enhancing allergen uptake by APCs, acting as an allergen depot, presenting a higher number of copies of the antigens to the individual APC, and causing the presence of the adjuvant and allergen within the same APC.

We found that the observed IgE levels after exposure to PSP and OVA were explained far better by the total surface area and particle number than by particle mass. The importance of surface area in determining biological effects of particles is discussed in paper II. We cannot exclude that the higher adjuvant capacity of smaller particles is caused merely by an increased antigen dose carried on particles, as the larger particle surface area is able to adsorb more allergen. However, there are additional mechanistic explanations for the adjuvant effect of particles that are discussed below.

In our study, all particle samples were mixed with allergen 1-18 h before injection, making it possible for allergen to adsorb to the particle surfaces. De Haar and colleagues even demonstrated that the amount of OVA bound to PSP increased drastically with decreasing particle size (de Haar, 2006). Indeed, co-administration of particles and allergen seems important in the footpad injection model using Balb/cA mice (Nygaard, 2005).

6.2. Particles directly influence the immune system

However, the adjuvant effect of particles appears not to be solely linked to the capacity of particles to act as allergen carriers. It has previously been demonstrated that even though ultrafine TiO_2 had much less allergen-binding capacity than ultrafine CBP, only TiO_2 increased the IgE response to OVA in an intranasal model of immunisation (de Haar *et al.*, 2006). In addition, particles have in intranasal and intraperitoneal models been shown to enhance the allergen-specific IgE responses also when particles were given one day before the allergen (Granum *et al.*, 2001b; van Zijverden *et al.*, 2001). Moreover, particle effects on lymph node cells such as increased expression of cell surface molecules and *ex vivo* cytokine production, have previously been reported by our group for ambient air particles in Balb/cA mice and for 0.1 µm PSP in other mouse strains (Nygaard *et al.*, 2005b; Nygaard *et al.*, 2005a). To investigate the acute effects of particles during the very early stages of the immune response, the effect on innate immune cell activation was studied in the *Listeria* challenge model.

6.2.1 Particles activate cells of the innate immune system

In paper III and IV we found that chemical-free model particles, as well as particles from wood smoke and road traffic activate cells of the innate immune system seen in the *Listeria* challenge model as a reduction in bacterial numbers. Activation of phagocytic cells has previously been observed after short time inhalation of silica particles in rats (Antonini *et al.*, 2000a).

Impaired human and rat AM phagocytic activity have previously been reported after both *ex vivo* and *in vitro* exposure to DEP (Barlow *et al.*, 2007; Lundborg *et al.*, 2006; Yin *et al.*, 2007). We have not in our study investigated a possible suppressive effect of particles on the phagocytic activity of AMs. However, theoretically, even with some degree of phagocytic suppression, activated macrophages might be able to reduce the number of *Listeria* by

endocytic killing compared to non-activated macrophages in which *Listeria* replicate by escaping into the cytosol as discussed in section 5.3.2. An *In vitro* study have suggested that the capacity of AMs to kill *Listeria* during the first 18 h after bacterial challenge was reduced when the AMs had been pre-exposed to DEP for 24 h (Yin *et al.*, 2007), as opposed to what we observed *in vivo*. However, since we studied the functional effect of particles on the intact pulmonary innate immune system, this discrepancy may be explained by the interaction of different cells and mediators in our exposure model.

The *Listeria* challenge model does not reveal which cells are involved in the observed innate immune response, and the specific activation of the different cells in the airways requires further investigation. Still, upon activation it is likely that all innate immune cells are affected, directly or as bystandards. Exposure to the model particle DEP have indeed been shown to influence cytokine production by, as well as the activation and function of phagocytic, epithelial and dendritic cells as described in section 2.4.1. Activation of macrophages and DCs, which act as APCs, is important in linking the innate and adaptive immune system (section 2.2.3.). These cells are essential in allergic sensitisation since APCs upon activation and migration to lymph nodes are able to activate naive T-cells as described in section 2.3.3. It has recently been suggested that DEP exposure indirectly induces functional activation of immature monocyte derived DCs via their influence on epithelial cells (Bleck *et al.*, 2008). Moreover, our lab has previously found particles localised within APCs in the PLN after footpad injection (Nygaard *et al.*, 2005b). Therefore, the capacity of particles to activate cells of the innate immune system is thought to be important also in relation to their adjuvant activity.

6.2.2. The mechanisms behind particle induced cellular activation may be important also for the adjuvant effect of particles

In paper III we found that large (4.646 μm) and small (0.202 and 0.0588 μm) PSP activated the innate immune system, while the intermediate-sized 1.053 μm PSP did not. The observed biphasic particle effect made us hypothesise that coarse and ultrafine particles induce cellular activation via different mechanisms. This hypothesis was supported by the different kinetics in reduction of bacterial numbers, and different pattern of pro-inflammatory mediators observed in BAL fluid after exposure to the different-sized PSP. As discussed in section 6.1.2., we also observed that the adjuvant capacity of particles increased with decreasing particle size, small (0.202 and 0.0588 μm) PSP by far having the highest adjuvant

capacity per mass compared to PSP larger than 1 μm. Based on these results, we propose that not only the capacity of particles to activate innate immune cells, but rather the very mechanism behind this activation, may be important for the adjuvant effect of particles on allergic sensitisation.

6.2.2.1. Inflammation

The adjuvant effect of particles on allergic sensitisation has previously been linked to their capacity to cause inflammation in the lung (de Haar *et al.*, 2005; de Haar *et al.*, 2006). Inflammation may be an important mechanism behind the adjuvant effect exerted on antibody production by particles, since a large number of antigen-presenting and inflammatory cells will be present at the time and place of allergen entry. Moreover, it has been suggested that particles induce enhanced APC function and maturation (Bleck *et al.*, 2008; de Haar *et al.*, 2008; Porter *et al.*, 2007). However, all the different-sized PSP caused some degree of acute lung inflammation, observed as an increased influx of neutrophils in the lungs (paper III). Still, mainly ultrafine and fine (0.0588 and 0.202 μm) PSP enhanced allergic sensitisation. The number of neutrophils reflected neither the observed general innate immune cell activation, nor the adjuvant effect of any of the particles tested (paper III and IV). Based on our data, we can not exclude that the capacity of particles to induce lung inflammation is important for their adjuvant effect. It is plausible that in our model, neutrophil influx was not a sensitive marker of inflammatory capacity, and possible differences between the various particles might not have been detected. This notion is also supported by a very large variance in these data. However, our data do suggest that the mechanisms driving the inflammation are important, and that not all inflammation necessarily will have an adjuvant effect, as discussed below.

6.2.2.2. Cell surface receptors

Concerning both cellular activation and adjuvant effect of particles there seemed to be a critical change in particle effect between particle sizes of 0.202 μm and 1.053 μm. As discussed in paper III, fine and coarse particles are more readily taken up by receptor mediated phagocytosis, and might exert their effects via receptor mediated pathways, perhaps through an early increase in TNF-α levels. Un-opsonised particles about 1 μm in size have been shown to bind to scavenger receptors (Palecanda *et al.*, 1999), and such receptors have been suggested to limit DC migration and allergic airway inflammation (Arredouani *et al.*, 2007), which may explain the lack of adjuvant effect on allergic sensitization by the 1.053 μm

PSP particles. Differences in effects observed between 4.646 µm and 1.053 µm PSP may be caused by different binding capacity for surface receptors on macrophages, such as the above mentioned scavenger receptors.

Ultrafine-sized ceramic (ZrO_2) and metallic (TiO_2) particles, on the other hand, have been shown to upregulate the expression of various TLRs in human macrophages *in vitro* (Lucarelli *et al.*, 2004). Moreover, TLR-4 have been proposed to play a role in DEP induced airway inflammation (Inoue *et al.*, 2006). Previously, the activation of TLR-4 has been suggested to be required for optimal development of Th2 responses (Dabbagh *et al.*, 2002). The interaction of ultrafine particles with TLRs provides a possible link between innate immune cell activation and allergic sensitisation. Several innate immune cells, including DCs, express TLRs which may lead to the activation of Th cells and skew the immunologic reaction towards a Th2 response as outlined in section 2.2.3.

6.2.2.3. Oxidative stress

Another plausible mechanism behind the cellular activation induced by the smallest PSP and DEP is the reported high potential of various ultrafine particles to induce oxidative stress (Brown *et al.*, 2001; Donaldson *et al.*, 2001; Li *et al.*, 2008) as discussed in paper III. Particles with a diameter less than 0.2 µm have been shown to gain access to intracellular compartments by simple diffusion and lead to activation of promoters and gene-transcription through the production of oxidative stress. Increased production of reactive oxygen and nitrogen intermediates is an important mechanism of reducing the number of *Listeria* (Ohya *et al.*, 1998; Shiloh *et al.*, 1999). Antonini and colleagues suggested that up-regulation in the production of antimicrobial oxidants after exposure to silica particles was likely to be responsible for the enhanced clearance of *Listeria* three days after bacterial inoculation (Antonini *et al.*, 2000a). The hypothesis of increased ROS production is in line with experimental data indicating that the adjuvant effect of ultrafine particles is linked to the capacity of these particles to induce oxidative stress (Dong *et al.*, 2005; Wan and Diaz-Sanchez, 2007a; Whitekus *et al.*, 2002). Due to their small size, these particles may diffuse into the interstitial lung space, increasing their interaction with APCs. It has been suggested that the generation of oxidative stress at the APC level favours Th2 skewing of the immune response, while suppressing Th1 differentiation (Murata *et al.*, 2002; Peterson *et al.*, 1998). Not only ultrafine particle size, but also associated chemicals have been shown to increase ROS-production (Ma and Ma, 2002; Shima *et al.*, 2006; Xiao *et al.*, 2003), which could be one explanation for the higher adjuvant activity of DEP compared to CBP (paper I). The

importance of oxidative stress is strengthened by studies showing that particle-induced inflammatory responses *in vitro* can be partially inhibited by antioxidants (Dick *et al.*, 2003; Wan and Diaz-Sanchez, 2007b). Another study demonstrated that also the adjuvant effect of DEP on allergen-specific IgE production could be inhibited by antioxidants *in vivo* (Whitekus *et al.*, 2002). However, we did not include in our BAL fluid analysis any marker of ROS activity, and the capacity of the tested particles to induce production of oxidative stress in our exposure model needs to be further investigated.

6.2.2.4. Toxicity

Wood smoke particles were the only particles to show signs of cytotoxicity by increasing the level of LDH. The wood smoke sample contained relatively high amounts of PAH's and zink (paper IV), which may give cellular damage (Adamson *et al.*, 2000; Kubatova *et al.*, 2004) as discussed in paper IV. It has previously been suggested that particle induced airway damage may play a role in the adjuvant activity of air pollution (de Haar *et al.*, 2005). Some of the cytosolic constituents released from necrotic cells posses immune-stimulating potential by inducing DC maturation and are therefore regarded as endogenous adjuvants. When cells of the airways are damaged, the damaged cells themselves or endogenous material leaking into the airspace function as danger signals to the innate immune system (Gallucci and Matzinger 2001). Such danger signals have been shown to cause influx of neutrophils (Gallucci *et al.*, 1999) and function as endogenous adjuvants by inducing maturation and activation of DCs (Shi *et al.*, 2003a). Thus activation of innate immune cells might be induced by toxicity, and possibly explain the inflammatory response seen as neutrophil influx observed after exposure to wood smoke.

6.3. Which particle characteristics are important in deciding the capacity of particles to cause adjuvant effects?

6.3.1. Size versus chemical composition

Particle size seems to be an important factor in considering the effect of particles from wood smoke and road traffic on the innate immune system and allergic sensitisation, as discussed in paper I and IV. The response after DEP exposure resembled that of ultrafine PSP with rapid reduction in bacterial numbers, and no increase of TNF-α levels in BAL fluid.

DEP had in addition the highest adjuvant capacity of the particles tested on the IgE response to OVA. Exposure to mixed road traffic particles on the other hand, reflected the contribution of both coarse and ultrafine particles, leading to a less rapid reduction of bacterial numbers, increased TNF-α levels in BAL fluid, and a more moderate adjuvant effect. The response seen after exposure to wood smoke particles, however, could not be explained by the ultrafine primary particle size. No reduction in the number of bacteria was observed after simultaneous exposure to wood smoke particles and *Listeria*, and the adjuvant effect was significantly lower than after exposure to DEP and CBP. Differences in the patterns of inflammatory responses after exposure to wood smoke particles and DEP have previously been observed by others *in vivo* (Seagrave *et al.*, 2005). The explanation may reside in differences in chemical composition between wood smoke particles and DEP, like amount and types of PAHs, antioxidants and metals as discussed in paper I and IV. The importance of associated chemicals and metals, as well as organics such as endotoxin as modifiers of the biological effects of particles also applies to the mixed road traffic particles, including DEP, as discussed in paper I and IV.

6.3.2. Particles from vehicle exhaust versus wood smoke

Our results points to diesel engine generated particles as potentially the most harmful particles to respiratory health of those tested. In accordance with our results, epidemiologic studies suggest that vehicle emissions, and in particular heavy traffic, is associated with allergic sensitisation and aggravation of respiratory symptoms (de Kok *et al.*, 2006; Janssen *et al.*, 2003; Morgenstern *et al.*, 2008). It is however, important to note that the DEP used in our studies is not a representative of contemporary diesel emissions, as discussed in section 5.1.1., and different types of DEP have been shown to induce different responses (Alberg, personal communication; Seagrave *et al.*, 2002; Singh *et al.*, 2004). In our model, wood smoke particles and mixed road traffic particles had similar capacity to enhance allergic sensitisation, and traffic-related particles have previously been associated with adverse health effects (Brugge *et al.*, 2007; Brunekreef and Holgate, 2002; Heinrich and Wichmann, 2004). Efforts are already being made to reduce vehicle emissions and the use of low sulphur fuel and a catalysed trap has been shown to greatly reduce the toxic effect of diesel emissions (McDonald *et al.*, 2004). However, as mentioned in section 2.1.2., in some urban areas wood smoke particles make a substantial contribution to the already high concentrations of ambient

air particles. Based on our results we suggest that reduced emissions of particles from wood smoke would be an effective approach to reduce adverse health effects.

6.4. Limitations of the study and future perspectives

Limitations to our work include that the studies of particle induced innate immune responses and adjuvant activity of particles are performed using two different exposure routes, namely lung and footpad, respectively. However, we believe that essentially the same immune mechanisms are involved in the footpad and in the lung. This is supported by similar patterns of adjuvant effects on allergic sensitisation after footpad injection and intranasal exposure to PSPs of different sizes (Alberg, unpublished results). Additionally, indications of inflammatory responses have also been detected in the PLN, observed as increased cell number and cell proliferation after particle exposure. However, since there are obvious differences between footpad and airway immunisation, as discussed in section 5.2.1., the adjuvant effect of the combustion particle containing samples should also be tested in an airway model of allergic sensitisation, exposure by inhalation being the natural route.

Our indications of particle effects being mediated by different mechanisms depending on size and chemical composition calls for more in-depth mechanistic studies, with focus on the capacity of the different particles to induce oxidative stress in our lung exposure model as well as TNF-α mediated effects. The duration of the particle effects on innate immune responses is also relevant, especially since preliminary results indicate that DEP and wood smoke particles have more persistent effects than mixed road traffic particles. Moreover, since BAL fluid does not accurately reflect the immune responses in the lung tissue and airway surface mucosa, histopathological methods should preferentially be included. Future investigations should also include studies of how the various particles affect the different cells involved in the innate immune response, with focus on the APC's, and particularly DC's which are central in the development of allergic sensitisation.

To be able to draw more general conclusions on the health damaging capacity of different types of combustion particles present in ambient air, it will be necessary to include a broader spectre of relevant particle samples from the burning of wood and vehicle emission, both directly from the source and in ambient air after the influence of atmospheric chemistry.

7. Conclusion

In this work we found that particles from wood smoke and road traffic had the capacity to increase the development of allergic immune responses. We also found that this capacity seemed to depend on particle size. Based on our data, we suggest that it is mainly the fine and ultrafine particle fractions of ambient PM, primarily consisting of combustion generated particles, which are responsible for the adjuvant effects. Associated chemicals modulated these effects either by enhancing the adjuvant particle effects as observed for DEP compared to CBP, or decrease the adjuvant effect as seemed to be the case for wood smoke particles. The chemicals responsible, however, remain to be identified.

Similar conclusions seemed also to apply for the capacity of particles to activate innate immune cells and induce inflammation in the lung. Even though both coarse and fine/ultrafine particles (<0.2 μm) induced cellular activation, the mechanisms behind this activation seemed to be different and to be dependent on particle size. Both for the capacity of particles to activate innate immune cells and increase allergic sensitisation there seemed to be a distinction between 1 and 0.2 μm. This led us to hypothesise that not only the capacity of particles to induce inflammation, but rather the mechanisms behind particle induced cellular activation and inflammation might be important for the capacity of particles to increase allergic sensitisation, perhaps through the activation of APCs. While cellular activation by coarse particles seemed to involve an early increase in TNF-α, other mechanisms might be involved in activation by fine/ultrafine particles smaller than 0.2 μm, generation of oxidative stress being a plausible candidate. Since particle effects on human pulmonary health in general by many have been linked to the fine and ultrafine PM fraction, the mechanisms behind cellular activation caused by these particles may also be involved in the development of other adverse cardiopulmonary health effects.

Consequently, reducing the emissions of the fine and ultrafine ambient PM fractions, particularly ultrafine particles from traffic powered by diesel, but also wood smoke particles in heavily exposed areas, will be important means to reduce adverse pulmonary health effects from ambient air pollution.

8. References

Adamson, I. Y., Prieditis, H., Hedgecock, C., and Vincent, R. (2000). Zinc is the toxic factor in the lung response to an atmospheric particulate sample. *Toxicol. Appl. Pharmacol.* **166,** 111-119.

Akbari, O., Stock, P., Meyer, E., Kronenberg, M., Sidobre, S., Nakayama, T., Taniguchi, M., Grusby, M. J., Dekruyff, R. H., and Umetsu, D. T. (2003). Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat. Med.* **9,** 582-588.

Akira, S. (2003). Mammalian Toll-like receptors. *Curr. Opin. Immunol.* **15,** 5-11.

Akira, S., Uematsu, S., and Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell* **124,** 783-801.

Alfaro-Moreno, E., Nawrot, T. S., Nemmar, A., and Nemery, B. (2007). Particulate matter in the environment: pulmonary and cardiovascular effects. *Curr. Opin. Pulm. Med.* **13,** 98-106.

Anderson, M. E., and Bogdan, G. M. (2007). Environments, indoor air quality, and children. *Pediatr. Clin. North Am.* **54,** 295-307.

Annesi-Maesano, I., Moreau, D., Caillaud, D., Lavaud, F., Le Moullec, Y., Taytard, A., Pauli, G., and Charpin, D. (2007). Residential proximity fine particles related to allergic sensitisation and asthma in primary school children. *Respir. Med.* **101,** 1721-1729.

Antonini, J. M., Roberts, J. R., Yang, H. M., Barger, M. W., Ramsey, D., Castranova, V., and Ma, J. Y. (2000a). Effect of silica inhalation on the pulmonary clearance of a bacterial pathogen in Fischer 344 rats. *Lung* **178,** 341-350.

Antonini, J. M., Yang, H. M., Ma, J. Y., Roberts, J. R., Barger, M. W., Butterworth, L., Charron, T. G., and Castranova, V. (2000b). Subchronic silica exposure enhances respiratory defense mechanisms and the pulmonary clearance of Listeria monocytogenes in rats. *Inhal. Toxicol.* **12,** 1017-1036.

Applequist, S. E., Wallin, R. P., and Ljunggren, H. G. (2002). Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines. *Int. Immunol.* **14,** 1065-1074.

Armitage, R. J., Macduff, B. M., Spriggs, M. K., and Fanslow, W. C. (1993). Human B cell proliferation and Ig secretion induced by recombinant CD40 ligand are modulated by soluble cytokines. *J. Immunol.* **150,** 3671-3680.

Arredouani, M. S., Franco, F., Imrich, A., Fedulov, A., Lu, X., Perkins, D., Soininen, R., Tryggvason, K., Shapiro, S. D., and Kobzik, L. (2007). Scavenger Receptors SR-AI/II and MARCO limit pulmonary dendritic cell migration and allergic airway inflammation. *J. Immunol.* **178,** 5912-5920.

Bacharier, L. B., and Geha, R. S. (2000). Molecular mechanisms of IgE regulation. *J. Allergy Clin. Immunol.* **105,** S547-S558.

Banchereau, J., and Steinman, R. M. (1998). Dendritic cells and the control of immunity. *Nature* **392,** 245-252.

Barlow, P. G., Brown, D. M., Donaldson, K., MacCallum, J., and Stone, V. (2008). Reduced alveolar macrophage migration induced by acute ambient particle (PM(10)) exposure. *Cell Biol. Toxicol.* **24**, 243-252.

Barrett, E. G., Henson, R. D., Seilkop, S. K., McDonald, J. D., and Reed, M. D. (2006). Effects of hardwood smoke exposure on allergic airway inflammation in mice. *Inhal. Toxicol.* **18,** 33-43.

Baxter, D. W., and Port, C. C. (1974). Large-volume intratracheal instillation of particle suspensions to hamsters. In Experimental lung cancer (E.Karbe and J.F.Park, Eds.), pp. 86-91. Springer-Verlag, New York.

Beasley, R., Crane, J., Lai, C. K., and Pearce, N. (2000). Prevalence and etiology of asthma. *J. Allergy Clin. Immunol.* **105,** S466-S472.

Becker, S., Mundandhara, S., Devlin, R. B., and Madden, M. (2005). Regulation of cytokine production in human alveolar macrophages and airway epithelial cells in response to ambient air pollution particles: Further mechanistic studies. *Toxicol. Appl. Pharmacol.* **207,** 269-275.

Becker, S., and Soukup, J. (2003). Coarse(PM(2.5-10)), fine(PM(2.5)), and ultrafine air pollution particles induce/increase immune costimulatory receptors on human blood-derived monocytes but not on alveolar macrophages. *J. Toxicol. Environ. Health A* **66,** 847-859.

Behndig, A. F., Mudway, I. S., Brown, J. L., Stenfors, N., Helleday, R., Duggan, S. T., Wilson, S. J., Boman, C., Cassee, F. R., Frew, A. J., Kelly, F. J., Sandstrom, T., and Blomberg, A. (2006). Airway antioxidant and inflammatory responses to diesel exhaust exposure in healthy humans. *Eur. Respir. J.* **27,** 359-365.

Bennett, W. D., and Brown, J. S. (2005). Particulate dosimetry in the respiratory tract. In Air pollutants and the respiratory tract (W.M.Foster and D.L.Costa, Eds.), 2nd ed., pp. 21-73. Taylor & Francis Group.

Bermudez, E., Mangum, J. B., Wong, B. A., Asgharian, B., Hext, P. M., Warheit, D. B., and Everitt, J. I. (2004). Pulmonary responses of mice, rats, and hamsters to subchronic inhalation of ultrafine titanium dioxide particles. *Toxicol. Sci.* **77,** 347-357.

BeruBe, K., Balharry, D., Sexton, K., Koshy, L., and Jones, T. (2007). Combustion-derived nanoparticles: mechanisms of pulmonary toxicity. *Clin. Exp. Pharmacol. Physiol.* **34,** 1044-1050.

Bilyk, N., and Holt, P. G. (1995). Cytokine modulation of the immunosuppressive phenotype of pulmonary alveolar macrophage populations. *Immunology* **86,** 231-237.

Bjorksten, B., Naaber, P., Sepp, E., and Mikelsaar, M. (1999). The intestinal microflora in allergic Estonian and Swedish 2-year-old children. *Clin. Exp. Allergy* **29,** 342-346.

Bleck, B., Tse, D. B., Curotto de Lafaille, M. A., Zhang, F., and Reibman, J. (2008). Diesel Exhaust Particle-Exposed Human Bronchial Epithelial Cells Induce Dendritic Cell Maturation and Polarization via Thymic Stromal Lymphopoietin. *J. Clin. Immunol.* **28,** 147-156.

Bleck, B., Tse, D. B., Jaspers, I., Curotto de Lafaille, M. A., and Reibman, J. (2006). Diesel exhaust particle-exposed human bronchial epithelial cells induce dendritic cell maturation. *J. Immunol.* **176,** 7431-7437.

Boman, B. C., Forsberg, A. B., and Jarvholm, B. G. (2003). Adverse health effects from ambient air pollution in relation to residential wood combustion in modern society. *Scand. J. Work Environ. Health* **29,** 251-260.

Brauer, M., Avila-Casado, C., Fortoul, T. I., Vedal, S., Stevens, B., and Churg, A. (2001). Air pollution and retained particles in the lung. *Environ. Health Perspect.* **109,** 1039-1043.

Brauer, M., Hoek, G., Smit, H. A., de Jongste, J. C., Gerritsen, J., Postma, D. S., Kerkhof, M., and Brunekreef, B. (2007). Air pollution and development of asthma, allergy and infections in a birth cohort. *Eur. Respir. J.* **29,** 879-888.

Braun-Fahrlander, C. (2003). Environmental exposure to endotoxin and other microbial products and the decreased risk of childhood atopy: evaluating developments since April 2002. *Curr. Opin. Allergy Clin. Immunol.* **3,** 325-329.

Brook, R. D., Franklin, B., Cascio, W., Hong, Y., Howard, G., Lipsett, M., Luepker, R., Mittleman, M., Samet, J., Smith, S. C., Jr., and Tager, I. (2004). Air pollution and cardiovascular disease: a statement for healthcare professionals from the Expert Panel on Population and Prevention Science of the American Heart Association. *Circulation* **109,** 2655-2671.

Brown, D. M., Wilson, M. R., MacNee, W., Stone, V., and Donaldson, K. (2001). Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. *Toxicol. Appl. Pharmacol.* **175,** 191-199.

Brugge, D., Durant, J. L., and Rioux, C. (2007). Near-highway pollutants in motor vehicle exhaust: A review of epidemiologic evidence of cardiac and pulmonary health risks. *Environ. Health* **6,** 23.

Brunekreef, B., and Holgate, S. T. (2002). Air pollution and health. *Lancet* **360,** 1233-1242.

Byersdorfer, C. A., and Chaplin, D. D. (2001). Visualization of early APC/T cell interactions in the mouse lung following intranasal challenge. *J. Immunol.* **167,** 6756-6764.

Callard, R. E., and Harper, J. I. (2007). The skin barrier, atopic dermatitis and allergy: a role for Langerhans cells? *Trends Immunol.* **28,** 294-298.

Campbell, P. A. (1994). Macrophage-Listeria interactions. *Immunol. Ser.* **60,** 313-328.

Closs, O. (1975). Experimental murine leprosy: growth of Mycobacterium lepraemurium in C3H and C57/BL mice after footpad inoculation. *Infect. Immun.* **12,** 480-489.

Cohen, A. B., and Rossi, M. (1983). Neutrophils in normal lungs. *Am. Rev. Respir. Dis.* **127,** S3-S9.

Cook, D. N., and Bottomly, K. (2007). Innate immune control of pulmonary dendritic cell trafficking. *Proc. Am. Thorac. Soc.* **4,** 234-239.

Costa, D. L., Lehmann, J. R., Harold, W. M., and Drew, R. T. (1986). Transoral tracheal intubation of rodents using a fiberoptic laryngoscope. *Lab. Anim. Sci.* **36,** 256-261.

Cousens, L. P., and Wing, E. J. (2000). Innate defenses in the liver during Listeria infection. *Immunol. Rev.* **174,** 150-159.

Cox, J. C., and Coulter, A. R. (1997). Adjuvants--a classification and review of their modes of action. *Vaccine* **15,** 248-256.

Cuq, P. (1966). Le système lymphatique de la Souris. *Recl. Med. Vet.* **142,** 1211-1236.

Dabbagh, K., Dahl, M. E., Stepick-Biek, P., and Lewis, D. B. (2002). Toll-like receptor 4 is required for optimal development of Th2 immune responses: role of dendritic cells. *J Immunol* **168,** 4524-4530.

de Haar, C. Particulate matter-induced allergic airway sensitization. (2006). Institute for Risk Assesment Sciences (IRAS), Utrecht University, Netherlands.

de Haar, C., Hassing, I., Bol, M., Bleumink, R., and Pieters, R. (2005). Ultrafine carbon black particles cause early airway inflammation and have adjuvant activity in a mouse allergic airway disease model. *Toxicol. Sci.* **87,** 409-418.

de Haar, C., Hassing, I., Bol, M., Bleumink, R., and Pieters, R. (2006). Ultrafine but not fine particulate matter causes airway inflammation and allergic airway sensitization to co-administered antigen in mice. *Clin. Exp. Allergy* **36,** 1469-1479.

de Haar, C., Kool, M., Hassing, I., Bol, M., Lambrecht, B. N., and Pieters, R. (2008). Lung dendritic cells are stimulated by ultrafine particles and play a key role in particle adjuvant activity. *J. Allergy Clin. Immunol*. **121**, 1246-1254.

de Kok, T. M., Driece, H. A., Hogervorst, J. G., and Briede, J. J. (2006). Toxicological assessment of ambient and traffic-related particulate matter: a review of recent studies. *Mutat. Res.* **613,** 103-122.

Devalia, J. L., Bayram, H., Rusznak, C., Calderon, M., Sapsford, R. J., Abdelaziz, M. A., Wang, J., and Davies, R. J. (1997). Mechanisms of pollution-induced airway disease: in vitro studies in the upper and lower airways. *Allergy* **52,** 45-51.

Devereux, G. (2006). The increase in the prevalence of asthma and allergy: food for thought. *Nat. Rev. Immunol.* **6,** 869-874.

Devouassoux, G., Saxon, A., Metcalfe, D. D., Prussin, C., Colomb, M. G., Brambilla, C., and Diaz-Sanchez, D. (2002). Chemical constituents of diesel exhaust particles induce IL-4 production and histamine release by human basophils. *J. Allergy Clin. Immunol.* **109,** 847-853.

Diaz-Sanchez, D., Garcia, M. P., Wang, M., Jyrala, M., and Saxon, A. (1999). Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. *J. Allergy Clin. Immunol.* **104,** 1183-1188.

Diaz-Sanchez, D., Jyrala, M., Ng, D., Nel, A., and Saxon, A. (2000a). In vivo nasal challenge with diesel exhaust particles enhances expression of the CC chemokines rantes, MIP-1alpha, and MCP-3 in humans. *Clin. Immunol.* **97,** 140-145.

Diaz-Sanchez, D., Penichet-Garcia, M., and Saxon, A. (2000b). Diesel exhaust particles directly induce activated mast cells to degranulate and increase histamine levels and symptom severity. *J. Allergy Clin. Immunol.* **106,** 1140-1146.

Diaz-Sanchez, D., Tsien, A., Fleming, J., and Saxon, A. (1997). Combined diesel exhaust particulate and ragweed allergen challenge markedly enhances human in vivo nasal ragweed-specific IgE and skews cytokine production to a T helper cell 2-type pattern. *J. Immunol.* **158,** 2406-2413.

Dick, C. A., Brown, D. M., Donaldson, K., and Stone, V. (2003). The role of free radicals in the toxic and inflammatory effects of four different ultrafine particle types. *Inhal. Toxicol.* **15,** 39-52.

Dinarello, C. A. (2000). Proinflammatory cytokines. *Chest* **118,** 503-508.

Don Porto, C. A., Hoet, P. H., Nemery, B., and Schoeters, G. (2002). Increased HLA-DR expression after exposure of human monocytic cells to air particulates. *Clin. Exp. Allergy* **32,** 296-300.

Donaldson, K., Stone, V., Clouter, A., Renwick, L., and MacNee, W. (2001). Ultrafine particles. *Occup. Environ. Med.* **58,** 211-6, 199.

Donaldson, K., Stone, V., Gilmour, P. S., Brown, D. M., and MacNee, W. (2000). Ultrafine particles: mechanisms of lung injury. *Phil. Trans. R. Soc. Lond. A* **358,** 2741-2749.

Donaldson, K., Tran, L., Jimenez, L. A., Duffin, R., Newby, D. E., Mills, N., MacNee, W., and Stone, V. (2005). Combustion-derived nanoparticles: a review of their toxicology following inhalation exposure. *Part. Fibre Toxicol.* **2,** 10.

Dong, C. C., Yin, X. J., Ma, J. Y., Millecchia, L., Wu, Z. X., Barger, M. W., Roberts, J. R., Antonini, J. M., Dey, R. D., and Ma, J. K. (2005). Effect of diesel exhaust particles on allergic reactions and airway responsiveness in ovalbumin-sensitized brown Norway rats. *Toxicol. Sci.* **88,** 202-212.

Driscoll, K. E. (2000). TNFalpha and MIP-2: role in particle-induced inflammation and regulation by oxidative stress. *Toxicol. Lett.* **112-113,** 177-183.

Driscoll, K. E., Carter, J. M., Hassenbein, D. G., and Howard, B. (1997). Cytokines and particle-induced inflammatory cell recruitment. *Environ. Health Perspect.* **105**(Suppl 5), 1159-1164.

Driscoll, K. E., Costa, D. L., Hatch, G., Henderson, R., Oberdorster, G., Salem, H., and Schlesinger, R. B. (2000). Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. *Toxicol. Sci.* **55,** 24-35.

Dybing, E., Lovdal, T., Hetland, R. B., Lovik, M., and Schwarze, P. E. (2004). Respiratory allergy adjuvant and inflammatory effects of urban ambient particles. *Toxicology* **198,** 307-314.

Eisenbarth, S. C., Piggott, D. A., and Bottomly, K. (2003). The master regulators of allergic inflammation: dendritic cells in Th2 sensitization. *Curr. Opin. Immunol.* **15,** 620-626.

Elder, A., Gelein, R., Finkelstein, J. N., Driscoll, K. E., Harkema, J., and Oberdorster, G. (2005). Effects of subchronically inhaled carbon black in three species. I. Retention kinetics, lung inflammation, and histopathology. *Toxicol. Sci.* **88,** 614-629.

Fang, F. C. (2004). Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat. Rev. Microbiol.* **2,** 820-832.

Festing, M. F. W., Overend, P., Das, R. G., Borja, M. C., and Berdoy, M. (2002). The design of experiments. In The design of animal experiments; Reducingthe use of animals in research through better experimental design (B.Waynforth, Ed.), 1st ed., pp. 54-58. Laboratory Animals Ltd by The Royal Society of Medicine Press Limited, London.

Finkelman, F. D., Holmes, J., Katona, I. M., Urban, J. F., Jr., Beckmann, M. P., Park, L. S., Schooley, K. A., Coffman, R. L., Mosmann, T. R., and Paul, W. E. (1990). Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol.* **8,** 303-333.

Frampton, M. W. (2006). Inflammation and airborne particles. *Clin. Occup. Environ. Med.* **5,** 797-815.

Fujieda, S., Diaz-Sanchez, D., and Saxon, A. (1998). Combined nasal challenge with diesel exhaust particles and allergen induces In vivo IgE isotype switching. *Am. J. Respir. Cell Mol. Biol.* **19,** 507-512.

Gallucci, S., Lolkema, M., and Matzinger, P. (1999). Natural adjuvants: endogenous activators of dendritic cells. *Nat. Med.* **5,** 1249-1255.

Gallucci, S., and Matzinger, P. (2001). Danger signals: SOS to the immune system. *Curr. Opin. Immunol.* **13,** 114-119.

Gehr, P., Geiser, M., Im, H., V, and Schürch, S. (2000). Surfactant-ultrafine particle interactions: what we can learn from PM_{10} studies. *Phil. Trans. R.. Soc. Lond. A* **358,** 2707-2718.

Gekara, N. O., and Weiss, S. (2007). Mast cells initiate early anti-Listeria host defences. *Cell Microbiol*. **10,** 225-236.

Gelfand, E. W. (2004). Inflammatory mediators in allergic rhinitis. *J. Allergy Clin. Immunol.* **114,** S135-S138.

Gerhold, K., Blumchen, K., Bock, A., Franke, A., Avagjan, A., and Hamelmann, E. (2002). Endotoxins and allergy: lessons from the murine model. *Pathobiology* **70,** 255-259.

Ghio, A. J., Silbajoris, R., Carson, J. L., and Samet, J. M. (2002). Biologic effects of oil fly ash. *Environ. Health Perspect.* **110**(Suppl 1), 89-94.

Glasius, M., Ketzel, M., Wåhlin, P., Jensen, B., Mønster, J., Berkowicz, R., and Palmgren, F. (2006). Impact of wood combustion on particle levels in a residential area in Denmark. *Atmos. Environ.* **40,** 7115-7124.

Gor, D. O., Rose, N. R., and Greenspan, N. S. (2003). TH1-TH2: a procrustean paradigm. *Nat. Immunol.* **4,** 503-505.

Gould, H. J., and Sutton, B. J. (2008). IgE in allergy and asthma today. *Nat. Rev. Immunol.* **8,** 205-217.

Granum, B., Gaarder, P. I., and Lovik, M. (2000). IgE adjuvant activity of particles - what physical characteristics are important? *Inhal.Toxicol.* **12**(Suppl 3), 365-372.

Granum, B., Gaarder, P. I., Groeng, E., Leikvold, R., Namork, E., and Lovik, M. (2001a). Fine particles of widely different composition have an adjuvant effect on the production of allergen-specific antibodies. *Toxicol. Lett.* **118,** 171-181.

Granum, B., Gaarder, P. I., and Lovik, M. (2001b). IgE adjuvant effect caused by particles - immediate and delayed effects. *Toxicology* **156,** 149-159.

Granum, B., and Lovik, M. (2002). The effect of particles on allergic immune responses. *Toxicol. Sci.* **65,** 7-17.

Guo, R. F., and Ward, P. A. (2002). Mediators and regulation of neutrophil accumulation in inflammatory responses in lung: insights from the IgG immune complex model. *Free Radic. Biol. Med.* **33,** 303-310.

Haas, H., Falcone, F. H., Holland, M. J., Schramm, G., Haisch, K., Gibbs, B. F., Bufe, A., and Schlaak, M. (1999). Early interleukin-4: its role in the switch towards a Th2 response and IgE-mediated allergy. *Int. Arch. Allergy Immunol.* **119,** 86-94.

Hamilton, R. F., Jr., Holian, A., and Morandi, M. T. (2004). A comparison of asbestos and urban particulate matter in the in vitro modification of human alveolar macrophage antigen-presenting cell function. *Exp. Lung Res.* **30,** 147-162.

Hammad, H., and Lambrecht, B. N. (2007). Lung dendritic cell migration. *Adv. Immunol.* **93,** 265-278.

Hammad, H., and Lambrecht, B. N. (2008). Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat. Rev. Immunol.* **8,** 193-204.

Harmsen, A. G., Muggenburg, B. A., Snipes, M. B., and Bice, D. E. (1985). The role of macrophages in particle translocation from lungs to lymph nodes. *Science* **230,** 1277-1280.

Harrison, R. M., and Yin, J. (2000). Particulate matter in the atmosphere: which particle properties are important for its effects on health? *Sci. Total Environ.* **249,** 85-101.

Hawiger, D., Inaba, K., Dorsett, Y., Guo, M., Mahnke, K., Rivera, M., Ravetch, J. V., Steinman, R. M., and Nussenzweig, M. C. (2001). Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J. Exp. Med.* **194,** 769-779.

Heinrich, J., Mike, P., Wolfgang, B., Norbert, K., and Paul, J. A. P. (2003). Endotoxin in fine (PM 2.5) and coarse (PM 2.5-10) particle mass of ambient aerosols. A temporo-spatial analysis. *Atmos. Environ.* **37,** 3659-3667.

Heinrich, J., and Wichmann, H. E. (2004). Traffic related pollutants in Europe and their effect on allergic disease. *Curr. Opin. Allergy Clin. Immunol.* **4,** 341-348.

Heinrich, U., Fuhst, S., Rittinghausen, O., Creutzenberg, O., Bellmann, B., Koch, W., and Levsen, K. (1995). Chronic inhalation exposure of winstar rats and two different strains of mice to diesel engine exhaust, carbon black, and titanium dioxide. *Inhal. Toxicol.* **7,** 533-556.

Ho, K. F., Cao, J. J., Lee, S. C., and Chan, C. K. (2006). Source apportionment of PM2.5 in urban area of Hong Kong. *J. Hazard. Mater.* **138,** 73-85.

Hoebe, K., Janssen, E., and Beutler, B. (2004). The interface between innate and adaptive immunity. *Nat. Immunol.* **5,** 971-974.

Holgate, S. T., and Church, M. K. (1993). Allergy, Gower Medical Publishing, London.

Holt, P. G., Rose, A. H., Batty, J. E., and Turner, K. J. (1981). Induction of adjuvant-independent IgE responses in inbred mice: primary, secondary, and persistent IgE responses to ovalbumin and ovomucoid. *Int. Arch. Allergy Appl. Immunol.* **65,** 42-50.

Ibald-Mulli, A., Wichmann, H. E., Kreyling, W., and Peters, A. (2002). Epidemiological evidence on health effects of ultrafine particles. *J. Aerosol. Med.* **15,** 189-201.

Infante-Duarte, C., Horton, H. F., Byrne, M. C., and Kamradt, T. (2000). Microbial lipopeptides induce the production of IL-17 in Th cells. *J. Immunol.* **165,** 6107-6115.

Inoue, K., Takano, H., Yanagisawa, R., Hirano, S., Ichinose, T., Shimada, A., and Yoshikawa, T. (2006). The role of toll-like receptor 4 in airway inflammation induced by diesel exhaust particles. *Arch. Toxicol.* **80,** 275-279.

Instanes, C., Ormstad, H., Rydjord, B., Wiker, H. G., and Hetland, G. (2004). Mould extracts increase the allergic response to ovalbumin in mice. *Clin. Exp. Allergy* **34,** 1634-1641.

Iwamura, C., and Nakayama, T. (2008). Toll-like receptors in the respiratory system: their roles in inflammation. *Curr. Allergy Asthma Rep.* **8,** 7-13.

Jabara, H. H., Fu, S. M., Geha, R. S., and Vercelli, D. (1990). CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. *J. Exp. Med.* **172,** 1861-1864.

Janssen, N. A., Brunekreef, B., Van Vliet, P., Aarts, F., Meliefste, K., Harssema, H., and Fischer, P. (2003). The relationship between air pollution from heavy traffic and allergic sensitization, bronchial hyperresponsiveness, and respiratory symptoms in Dutch schoolchildren. *Environ. Health Perspect.* **111,** 1512-1518.

Kappos, A. D., Bruckmann, P., Eikmann, T., Englert, N., Heinrich, U., Hoppe, P., Koch, E., Krause, G. H., Kreyling, W. G., Rauchfuss, K., Rombout, P., Schulz-Klemp, V., Thiel, W. R., and Wichmann, H. E. (2004). Health effects of particles in ambient air. *Int. J. Hyg. Environ. Health* **207,** 399-407.

Katrinak, K. A., Rez, P., Perkes, P. R., and Buseck, P. R. (1993). Fractal geometry of carbonaceous aggregates from an urban aerosol. *Envion. Sci. Technol.* **27,** 539-547.

Kay, A. B. (2001). Allergy and allergic diseases. First of two parts. *N. Engl. J. Med.* **344,** 30-37.

Kidd, P. (2003). Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. *Altern. Med. Rev.* **8,** 223-246.

KleinJan, A., Vinke, J. G., Severijnen, L. W., and Fokkens, W. J. (2000). Local production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis patients. *Eur. Respir. J.* **15,** 491-497.

Kocbach, A., Johansen, B. V., Schwarze, P. E., and Namork, E. (2005). Analytical electron microscopy of combustion particles: a comparison of vehicle exhaust and residential wood smoke. *Sci. Total. Environ.* **346,** 231-243.

Kocbach, A., Li, Y., Yttri, K. E., Cassee, F. R., Schwarze, P. E., and Namork, E. (2006). Physicochemical characterisation of combustion particles from vehicle exhaust and residential wood smoke. *Part. Fibre Toxicol.* **3,** 1.

Kreyling, W. G., Möller, W., Semmler-Behnke, M., and Oberdorster, G. (2007). Particle dosimetry: Deposition and clearance from the respiratory tract and translocation towards extra-pulmonary sites. In *Particle toxicology* (K.Donaldson and P.Borm, Eds.), pp. 47-74. CRC Press, Taylor & Francis Group, New York.

Kreyling, W. G., Semmler-Behnke, M., and Moller, W. (2006). Ultrafine particle-lung interactions: does size matter? *J. Aerosol. Med.* **19,** 74-83.

Kubatova, A., Steckler, T. S., Gallagher, J. R., Hawthorne, S. B., and Picklo, M. J., Sr. (2004). Toxicity of wide-range polarity fractions from wood smoke and diesel exhaust particulate obtained using hot pressurized water. *Environ. Toxicol. Chem.* **23,** 2243-2250.

Lambert, A. L., Dong, W., Selgrade, M. K., and Gilmour, M. I. (2000). Enhanced allergic sensitization by residual oil fly ash particles is mediated by soluble metal constituents. *Toxicol. Appl. Pharmacol.* **165,** 84-93.

Lambrecht, B. N. (2001). Allergen uptake and presentation by dendritic cells. *Curr. Opin. Allergy Clin. Immunol.* **1,** 51-59.

Lambrecht, B. N. (2006). Alveolar macrophage in the driver's seat. *Immunity* **24,** 366-368.

Larche, M. (2007). Regulatory T cells in allergy and asthma. *Chest* **132,** 1007-1014.

Larsen, G. L., and Holt, P. G. (2000). The concept of airway inflammation. *Am. J. Respir. Crit. Care Med.* **162,** S2-S6.

Li, N., Hao, M., Phalen, R. F., Hinds, W. C., and Nel, A. E. (2003). Particulate air pollutants and asthma. A paradigm for the role of oxidative stress in PM-induced adverse health effects. *Clin. Immunol.* **109,** 250-265.

Li, N., Xia, T., and Nel, A. E. (2008). The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. *Free Radic. Biol. Med.* **44,** 1689-1699.

Lighty, J. S., Veranth, J. M., and Sarofim, A. F. (2000). Combustion aerosols: factors governing their size and composition and implications to human health. *J. Air Waste Manag. Assoc.* **50,** 1565-1618.

Lipscomb, M. F., Pollard, A. M., and Yates, J. L. (1993). A role for TGF-beta in the suppression by murine bronchoalveolar cells of lung dendritic cell initiated immune responses. *Reg. Immunol.* **5,** 151-157.

Lipsett, M., Hurley, S., and Ostro, B. (1997). Air pollution and emergency room visits for asthma in Santa Clara County, California. *Environ. Health Perspect.* **105,** 216-222.

Lovik, M., Alberg, T., Nygaard, U. C., Samuelsen, M., Groeng, E. C., and Gaarder, P. I. (2007). Popliteal lymph node (PLN) assay to study adjuvant effects on respiratory allergy. *Methods* **41,** 72-79.

Lovik, M., Hogseth, A. K., Gaarder, P. I., Hagemann, R., and Eide, I. (1997). Diesel exhaust particles and carbon black have adjuvant activity on the local lymph node response and systemic IgE production to ovalbumin. *Toxicology* **121,** 165-178.

Lovik, M., and North, R. J. (1985). Effect of aging on antimicrobial immunity: old mice display a normal capacity for generating protective T cells and immunologic memory in response to infection with Listeria monocytogenes. *J. Immunol.* **135,** 3479-3486.

Lucarelli, M., Gatti, A. M., Savarino, G., Quattroni, P., Martinelli, L., Monari, E., and Boraschi, D. (2004). Innate defence functions of macrophages can be biased by nano-sized ceramic and metallic particles. *Eur. Cytokine Netw.* **15,** 339-346.

Lundborg, M., Dahlen, S. E., Johard, U., Gerde, P., Jarstrand, C., Camner, P., and Lastbom, L. (2006). Aggregates of ultrafine particles impair phagocytosis of microorganisms by human alveolar macrophages. *Environ. Res.* **100,** 197-204.

Ma, J. Y., and Ma, J. K. (2002). The dual effect of the particulate and organic components of diesel exhaust particles on the alteration of pulmonary immune/inflammatory responses and metabolic enzymes. *J Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev.* **20,** 117-147.

MacLean, J. A., Xia, W., Pinto, C. E., Zhao, L., Liu, H. W., and Kradin, R. L. (1996). Sequestration of inhaled particulate antigens by lung phagocytes. A mechanism for the effective inhibition of pulmonary cell-mediated immunity. *Am. J. Pathol.* **148,** 657-666.

Mayer, A. K., and Dalpke, A. H. (2007). Regulation of local immunity by airway epithelial cells. *Arch. Immunol. Ther. Exp. (Warsz.)* **55,** 353-362.

McDonald, J. D., Harrod, K. S., Seagrave, J., Seilkop, S. K., and Mauderly, J. L. (2004). Effects of low sulfur fuel and a catalyzed particle trap on the composition and toxicity of diesel emissions. *Environ. Health Perspect.* **112,** 1307-1312.

McLachlan, J. B., Shelburne, C. P., Hart, J. P., Pizzo, S. V., Goyal, R., Brooking-Dixon, R., Staats, H. F., and Abraham, S. N. (2008). Mast cell activators: a new class of highly effective vaccine adjuvants. *Nat. Med.* **14,** 536-541.

McWilliam, A. S., Holt, P. G., and Gehr, P. (2000). Dendritic cells as sentinels of immune surveillance in the airways. In Particle-Lung Interaction (P.Gehr and J.Heyder, Eds.), pp. 291-322. Marcel Dekker, New York.

Melgert, B. N., Postma, D. S., Kuipers, I., Geerlings, M., Luinge, M. A., van der Strate, B. W., Kerstjens, H. A., Timens, W., and Hylkema, M. N. (2005). Female mice are more susceptible to the development of allergic airway inflammation than male mice. *Clin. Exp. Allergy* **35,** 1496-1503.

Menache, M. G., Miller, F. J., and Raabe, O. G. (1995). Particle inhalability curves for humans and small laboratory animals. *Ann. Occup. Hyg.* **39,** 317-328.

Miyabara, Y., Takano, H., Ichinose, T., Lim, H. B., and Sagai, M. (1998). Diesel exhaust enhances allergic airway inflammation and hyperresponsiveness in mice. *Am. J. Respir. Crit. Care Med.* **157,** 1138-1144.

Moller, W., Barth, W., Kohlhaufl, M., Haussinger, K., Stahlhofen, W., and Heyder, J. (2001). Human alveolar long-term clearance of ferromagnetic iron oxide microparticles in healthy and diseased subjects. *Exp. Lung Res.* **27,** 547-568.

Morgenstern, V., Zutavern, A., Cyrys, J., Brockow, I., Koletzko, S., Kramer, U., Behrendt, H., Herbarth, O., von Berg, A., Bauer, C. P., Wichmann, H. E., and Heinrich, J. (2008). Atopic diseases, allergic sensitization, and exposure to traffic-related air pollution in children. *Am. J. Respir. Crit. Care Med.* **177,** 1331-1337.

Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., and Coffman, R. L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* **136,** 2348-2357.

Murata, Y., Shimamura, T., and Hamuro, J. (2002). The polarization of T(h)1/T(h)2 balance is dependent on the intracellular thiol redox status of macrophages due to the distinctive cytokine production. *Int. Immunol.* **14,** 201-212.

Murphy, K., Travers, P., and Walport, M. (2008). Janeway's immuno biology, Garland Science, Taylor & Francis Group, New York.

Murphy, S. A., BeruBe, K. A., Pooley, F. D., and Richards, R. J. (1998). The response of lung epithelium to well characterised fine particles. *Life Sci.* **62,** 1789-1799.

Naeher, L. P., Brauer, M., Lipsett, M., Zelikoff, J. T., Simpson, C. D., Koenig, J. Q., and Smith, K. R. (2007). Woodsmoke health effects: a review. *Inhal. Toxicol.* **19,** 67-106.

Namork, E., Johansen, B. V., and Lovik, M. (2006). Detection of allergens adsorbed to ambient air particles collected in four European cities. *Toxicol. Lett.* **165,** 71-78.

Nel, A. E., Diaz-Sanchez, D., Ng, D., Hiura, T., and Saxon, A. (1998). Enhancement of allergic inflammation by the interaction between diesel exhaust particles and the immune system. *J. Allergy Clin. Immunol.* **102,** 539-554.

Nelde, A., Teufel, M., Hahn, C., Duschl, A., Sebald, W., Brocker, E. B., and Grunewald, S. M. (2001). The impact of the route and frequency of antigen exposure on the IgE response in allergy. *Int. Arch. Allergy Immunol.* **124,** 461-469.

Nemmar, A., Hoet, P. H., Vanquickenborne, B., Dinsdale, D., Thomeer, M., Hoylaerts, M. F., Vanbilloen, H., Mortelmans, L., and Nemery, B. (2002). Passage of inhaled particles into the blood circulation in humans. *Circulation* **105,** 411-414.

Nicod, L. P., Cochand, L., and Dreher, D. (2000). Antigen presentation in the lung: dendritic cells and macrophages. *Sarcoidosis. Vasc. Diffuse. Lung Dis.* **17,** 246-255.

North, R. J. (1973). Cellular mediators of anti-Listeria immunity as an enlarged population of short lived, replicating T cells. Kinetics of their production. *J. Exp. Med.* **138,** 342-355.

Nygaard, U. C. The adjuvant effect of particles on the production of allergen-specific IgE - cells and mechanisms. (2005). Faculty of Mathematics and Natural Scineces, University of Oslo, Norway.

Nygaard, U. C., Aase, A., and Lovik, M. (2005a). The allergy adjuvant effect of particles - genetic factors influence antibody and cytokine responses. *BMC Immunol.* **6,** 11.

Nygaard, U. C., Alberg, T., Bleumink, R., Aase, A., Dybing, E., Pieters, R., and Lovik, M. (2005b). Ambient air particles from four European cities increase the primary cellular response to allergen in the draining lymph node. *Toxicology* **207,** 241-254.

Oberdorster, G., and Utell, M. J. (2002). Ultrafine particles in the urban air: to the respiratory tract--and beyond? *Environ. Health Perspect.* **110,** A440-A441.

Oboki, K., Ohno, T., Saito, H., and Nakae, S. (2008). Th17 and allergy. *Allergol. Int.* **57,** 121-134.

Ohya, S., Tanabe, Y., Makino, M., Nomura, T., Xiong, H., Arakawa, M., and Mitsuyama, M. (1998). The contributions of reactive oxygen intermediates and reactive nitrogen intermediates to listericidal mechanisms differ in macrophages activated pre- and postinfection. *Infect. Immun.* **66,** 4043-4049.

Ormstad, H., Johansen, B. V., and Gaarder, P. I. (1998). Airborne house dust particles and diesel exhaust particles as allergen carriers. *Clin. Exp. Allergy* **28,** 702-708.

Orozco-Levi, M., Garcia-Aymerich, J., Villar, J., Ramirez-Sarmiento, A., Anto, J. M., and Gea, J. (2006). Wood smoke exposure and risk of chronic obstructive pulmonary disease. *Eur. Respir. J.* **27,** 542-546.

Osier, M., and Oberdorster, G. (1997). Intratracheal inhalation vs intratracheal instillation: differences in particle effects. *Fundam. Appl. Toxicol.* **40,** 220-227.

Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L., and Aderem, A. (2000). The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc. Natl. Acad. Sci. USA* **97,** 13766-13771.

Palecanda, A., Paulauskis, J., Al Mutairi, E., Imrich, A., Qin, G., Suzuki, H., Kodama, T., Tryggvason, K., Koziel, H., and Kobzik, L. (1999). Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles. *J. Exp. Med.* **189,** 1497-1506.

Paoletti, L., De Berardis, B., and Diociaiuti, M. (2002). Physico-chemical characterisation of the inhalable particulate matter (PM10) in an urban area: an analysis of the seasonal trend. *Sci. Total Environ.* **292,** 265-275.

Parnia, S., Brown, J. L., and Frew, A. J. (2002). The role of pollutants in allergic sensitization and the development of asthma. *Allergy* **57,** 1111-1117.

Penttinen, P., Timonen, K. L., Tiittanen, P., Mirme, A., Ruuskanen, J., and Pekkanen, J. (2001). Ultrafine particles in urban air and respiratory health among adult asthmatics. *Eur. Respir. J.* **17,** 428-435.

Peters, A., Veronesi, B., Calderon-Garciduenas, L., Gehr, P., Chen, L. C., Geiser, M., Reed, W., Rothen-Rutishauser, B., Schurch, S., and Schulz, H. (2006). Translocation and potential neurological effects of fine and ultrafine particles a critical update. *Part. Fibre Toxicol.* **3,** 13.

Peters, A., Wichmann, H. E., Tuch, T., Heinrich, J., and Heyder, J. (1997). Respiratory effects are associated with the number of ultrafine particles. *Am. J. Respir. Crit. Care Med.* **155,** 1376-1383.

Peterson, J. D., Herzenberg, L. A., Vasquez, K., and Waltenbaugh, C. (1998). Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. *Proc. Natl. Acad. Sci. USA* **95,** 3071-3076.

Phalen, R. F. (2002). The Particulate Air Pollution Controversy: A Case Study and Lessons Learned, Kluwer Academic Publishers, London.

Pope, C. A., III, and Dockery, D. W. (2006). Health effects of fine particulate air pollution: lines that connect. *J. Air Waste. Manag. Assoc.* **56,** 709-742.

Porter, M., Karp, M., Killedar, S., Bauer, S. M., Guo, J., Williams, D., Breysse, P., Georas, S. N., and Williams, M. A. (2007). Diesel-enriched particulate matter functionally activates human dendritic cells. *Am. J. Respir. Cell Mol. Biol.* **37,** 706-719.

Poulsen, L. K. (2000). Regulation of the IgE allergic immune response by humoral and cellular factors. *APMIS* **Suppl 98,** 1-43.

Raabe, O. G. (1988). Regional deposition of inhaled monodisperse coarse and fine aerosol particles in small laboratory animals. In Inhaled particles VI (J.Dodgson, R.I.McCallum, M.R.Bailey, and D.R.Fisher, Eds.), pp. 53-63. Pergamon Press, Oxford.

Radauer, C., Bublin, M., Wagner, S., Mari, A., and Breiteneder, H. (2008). Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *J. Allergy Clin. Immunol.* **121,** 847-852.

Rao, K. M., Ma, J. Y., Meighan, T., Barger, M. W., Pack, D., and Vallyathan, V. (2005). Time course of gene expression of inflammatory mediators in rat lung after diesel exhaust particle exposure. *Environ. Health Perspect.* **113,** 612-617.

Reutershan, J., and Ley, K. (2004). Bench-to-bedside review: acute respiratory distress syndrome - how neutrophils migrate into the lung. *Crit. Care* **8,** 453-461.

Riedl, M., and Diaz-Sanchez, D. (2005). Biology of diesel exhaust effects on respiratory function. *J. Allergy Clin. Immunol.* **115,** 221-228.

Romagnani, S. (2001). TH1/TH2 Interleukins. In Cytokine reference (J.J.Oppenheim and M.Feldmann, Eds.), pp. 99-112. Academic Press, London.

Rowe, J., Heaton, T., Kusel, M., Suriyaarachchi, D., Serralha, M., Holt, B. J., de Klerk, N., Sly, P. D., and Holt, P. G. (2004). High IFN-gamma production by CD8+ T cells and early sensitization among infants at high risk of atopy. *J. Allergy Clin. Immunol.* **113,** 710-716.

Ruxton, G. D., and Colegrave, N. (2006). Different experimental designs. In Experimental design for the life sciences (G.D.Ruxton and N.Colegrave, Eds.), 2de ed., pp. 69-100. Oxford University Press Inc, New York.

Saber, A. T., Bornholdt, J., Dybdahl, M., Sharma, A. K., Loft, S., Vogel, U., and Wallin, H. (2005). Tumor necrosis factor is not required for particle-induced genotoxicity and pulmonary inflammation. *Arch. Toxicol.* **79,** 177-182.

Saber, A. T., Jacobsen, N. R., Bornholdt, J., Kjaer, S. L., Dybdahl, M., Risom, L., Loft, S., Vogel, U., and Wallin, H. (2006). Cytokine expression in mice exposed to diesel exhaust particles by inhalation. Role of tumor necrosis factor. *Part. Fibre Toxicol.* **3,** 4.

Salvi, S., Blomberg, A., Rudell, B., Kelly, F., Sandstrom, T., Holgate, S. T., and Frew, A. (1999). Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers. *Am. J. Respir. Crit. Care Med.* **159,** 702-709.

Saxon, A., and Diaz-Sanchez, D. (2000). Diesel exhaust as a model xenobiotic in allergic inflammation. *Immunopharmacology* **48,** 325-327.

Saxon, A., and Diaz-Sanchez, D. (2005). Air pollution and allergy: you are what you breathe. *Nat. Immunol.* **6,** 223-226.

Schafer, T., and Ring, J. (1997). Epidemiology of allergic diseases. *Allergy* **52,** 14-22.

Schreuder, A. B., Larson, T. V., Sheppard, L., and Claiborn, C. S. (2006). Ambient woodsmoke and associated respiratory emergency department visits in Spokane, Washington. *Int. J. Occup. Environ. Health* **12,** 147-153.

Schwarze, P., Kocbach, A., Herseth, J. I., and Sandstrom, T. (2008). Differential physicochemical properties of particles from wood combustion and traffic determine pro-inflammatory potential. *SOT 2008* **102**(1), 223(Abstract).

Schwarze, P. E., Ovrevik, J., Hetland, R. B., Becher, R., Cassee, F. R., Lag, M., Lovik, M., Dybing, E., and Refsnes, M. (2007). Importance of size and composition of particles for effects on cells in vitro. *Inhal. Toxicol.* **19**(Suppl 1)**,** 17-22.

Seagrave, J., McDonald, J. D., Gigliotti, A. P., Nikula, K. J., Seilkop, S. K., Gurevich, M., and Mauderly, J. L. (2002). Mutagenicity and in vivo toxicity of combined particulate and semivolatile organic fractions of gasoline and diesel engine emissions. *Toxicol. Sci.* **70,** 212-226.

Seagrave, J., McDonald, J. D., Reed, M. D., Seilkop, S. K., and Mauderly, J. L. (2005). Responses to subchronic inhalation of low concentrations of diesel exhaust and hardwood smoke measured in rat bronchoalveolar lavage fluid. *Inhal. Toxicol.* **17,** 657-670.

Segal, A. W. (2005). How neutrophils kill microbes. *Annu. Rev. Immunol.* **23,** 197-223.

Shaughnessy, L. M., and Swanson, J. A. (2007). The role of the activated macrophage in clearing Listeria monocytogenes infection. *Front. Biosci.* **12,** 2683-2692.

Shi, Y., Evans, J. E., and Rock, K. L. (2003a). Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425,** 516-521.

Shi, Z., Shao, L. Y., Jones, T. P., Whittaker, A. G., Lu, S. L., BeruBe, K. A., He, T., and Richards, R. J. (2003b). Characterization of airborne individual particles collected in an urban area, a satellite city and a clean air area in Beijing. *Atmos. Environ.* **37**, 4097-4108.

Shiloh, M. U., MacMicking, J. D., Nicholson, S., Brause, J. E., Potter, S., Marino, M., Fang, F., Dinauer, M., and Nathan, C. (1999). Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. *Immunity* **10,** 29-38.

Shima, H., Koike, E., Shinohara, R., and Kobayashi, T. (2006). Oxidative ability and toxicity of n-hexane insoluble fraction of diesel exhaust particles. *Toxicol. Sci.* **91,** 218-226.

Singh, P., DeMarini, D. M., Dick, C. A., Tabor, D. G., Ryan, J. V., Linak, W. P., Kobayashi, T., and Gilmour, M. I. (2004). Sample characterization of automobile and forklift diesel exhaust particles and comparative pulmonary toxicity in mice. *Environ. Health Perspect.* **112,** 820-825.

Steerenberg, P., Verlaan, A., de Klerk, A., Boere, A., Loveren, H., and Cassee, F. (2004a). Sensitivity to ozone, diesel exhaust particles, and standardized ambient particulate matter in rats with a listeria monocytogenes-induced respiratory infection. *Inhal. Toxicol.* **16,** 311-317.

Steerenberg, P. A., Withagen, C. E., van Dalen, W. J., Dormans, J. A., Cassee, F. R., Heisterkamp, S. H., and van Loveren, H. (2004b). Adjuvant activity of ambient particulate matter of different sites, sizes, and seasons in a respiratory allergy mouse model. *Toxicol. Appl. Pharmacol.* **200,** 186-200.

Steerenberg, P. A., Withagen, C. E., van Dalen, W. J., Dormans, J. A., Heisterkamp, S. H., van Loveren, H., and Cassee, F. R. (2005). Dose dependency of adjuvant activity of particulate matter from five European sites in three seasons in an ovalbumin-mouse model. *Inhal. Toxicol.* **17,** 133-145.

Steinmuller, C., Franke-Ullmann, G., Lohmann-Matthes, M. L., and Emmendorffer, A. (2000). Local activation of nonspecific defense against a respiratory model infection by application of interferon-gamma: comparison between rat alveolar and interstitial lung macrophages. *Am. J. Respir. Cell Mol. Biol.* **22,** 481-490.

Sternhufvud, C., Karvosenoja, N., Illerup, J., Kindbom, K., Lükewille, A., Johansson, M., and Jensen, D. (2004). Particulate matter emissions and abatement options in residential wood burning in the Nordic countries. Nordic Council of Ministers (NMR). pp. 1-72.

Stoeger, T., Reinhard, C., Takenaka, S., Schroeppel, A., Karg, E., Ritter, B., Heyder, J., and Schulz, H. (2006). Instillation of six different ultrafine carbon particles indicates a surface area threshold dose for acute lung inflammation in mice. *Environ. Health Perspect.* **114,** 328-333.

Strachan, D. P. (1989). Hay fever, hygiene, and household size. *BMJ* **299,** 1259-1260.

Strieter, R. M., Belperio, J. A., and Keane, M. P. (2002). Cytokines in innate host defense in the lung. *J. Clin. Invest.* **109,** 699-705.

Takabayshi, K., Corr, M., Hayashi, T., Redecke, V., Beck, L., Guiney, D., Sheppard, D., and Raz, E. (2006). Induction of a homeostatic circuit in lung tissue by microbial compounds. *Immunity* **24,** 475-487.

Takano, H., Yoshikawa, T., Ichinose, T., Miyabara, Y., Imaoka, K., and Sagai, M. (1997). Diesel exhaust particles enhance antigen-induced airway inflammation and local cytokine expression in mice. *Am. J. Respir. Crit. Care Med.* **156,** 36-42.

Takizawa, H. (2004). Diesel exhaust particles and their effect on induced cytokine expression in human bronchial epithelial cells. *Curr. Opin. Allergy. Clin. Immunol.* **4,** 355-359.

Tam, M. A., and Wick, M. J. (2004). Dendritic cells and immunity to Listeria: TipDCs are a new recruit. *Trends Immunol.* **25,** 335-339.

Tsien, A., Diaz-Sanchez, D., Ma, J., and Saxon, A. (1997). The organic component of diesel exhaust particles and phenanthrene, a major polyaromatic hydrocarbon constituent, enhances IgE production by IgE-secreting EBV-transformed human B cells in vitro. *Toxicol. Appl. Pharmacol.* **142,** 256-263.

Underhill, D. M., and Ozinsky, A. (2002). Phagocytosis of microbes: complexity in action. *Annu. Rev. Immunol.* **20,** 825-852.

Van Coillie, E., Van Damme, J., and Opdenakker, G. (1999). The MCP/eotaxin subfamily of CC chemokines. *Cytokine Growth Factor Rev.* **10,** 61-86.

van Loveren, H., Rombout, P. J., Wagenaar, S. S., Walvoort, H. C., and Vos, J. G. (1988). Effects of ozone on the defense to a respiratory Listeria monocytogenes infection in the rat. Suppression of macrophage function and cellular immunity and aggravation of histopathology in lung and liver during infection. *Toxicol. Appl. Pharmacol.* **94,** 374-393.

van Zijverden, M., de Haar, C., van Beelen, A., van Loveren, H., Penninks, A., and Pieters, R. (2001). Coadministration of antigen and particles optimally stimulates the immune response in an intranasal administration model in mice. *Toxicol. Appl. Pharmacol.* **177,** 174-178.

van Zijverden, M., and Granum, B. (2000). Adjuvant activity of particulate pollutants in different mouse models. *Toxicology* **152,** 69-77.

van Zijverden, M., van der, P. A., Bol, M., van Pinxteren, F. A., de Haar, C., Penninks, A. H., van Loveren, H., and Pieters, R. (2000). Diesel exhaust, carbon black, and silica particles display distinct Th1/Th2 modulating activity. *Toxicol. Appl. Pharmacol.* **168,** 131-139.

Vercelli, D. (2008). Discovering susceptibility genes for asthma and allergy. *Nat. Rev. Immunol.* **8,** 169-182.

Vercelli, D., Jabara, H. H., Arai, K., and Geha, R. S. (1989). Induction of human IgE synthesis requires interleukin 4 and T/B cell interactions involving the T cell receptor/CD3 complex and MHC class II antigens. *J. Exp. Med.* **169,** 1295-1307.

Vione, D., Maurino, V., Minero, C., Pelizzetti, E., Harrison, M. A., Olariu, R. I., and Arsene, C. (2006). Photochemical reactions in the tropospheric aqueous phase and on particulate matter. *Chem. Soc. Rev.* **35,** 441-453.

von Klot, S., Wolke, G., Tuch, T., Heinrich, J., Dockery, D. W., Schwartz, J., Kreyling, W. G., Wichmann, H. E., and Peters, A. (2002). Increased asthma medication use in association with ambient fine and ultrafine particles. *Eur. Respir. J.* **20,** 691-702.

Wan, J., and Diaz-Sanchez, D. (2007). Antioxidant enzyme induction: a new protective approach against the adverse effects of diesel exhaust particles. *Inhal. Toxicol.* **19**(Suppl 1) **,** 177-182.

Wang, Y., Bai, C., Li, K., Adler, K. B., and Wang, X. (2008). Role of airway epithelial cells in development of asthma and allergic rhinitis. *Respir. Med.* **102,** 949-955.

Whitekus, M. J., Li, N., Zhang, M., Wang, M., Horwitz, M. A., Nelson, S. K., Horwitz, L. D., Brechun, N., Diaz-Sanchez, D., and Nel, A. E. (2002). Thiol antioxidants inhibit the adjuvant effects of aerosolized diesel exhaust particles in a murine model for ovalbumin sensitization. *J. Immunol.* **168,** 2560-2567.

WHO. The world health report: reducing risks, promoting healthy life. (2002). WHO, Geneva, Switzerland.

Wittmaack, K. (2007). In search of the most relevant parameter for quantifying lung inflammatory response to nanoparticle exposure: particle number, surface area, or what? *Environ. Health Perspect.* **115,** 187-194.

Wu, C. F., Larson, T. V., Wu, S. Y., Williamson, J., Westberg, H. H., and Liu, L. J. (2007). Source apportionment of PM(2.5) and selected hazardous air pollutants in Seattle. *Sci. Total. Environ.* **386,** 42-52.

Xia, T., Kovochich, M., Brant, J., Hotze, M., Sempf, J., Oberley, T., Sioutas, C., Yeh, J. I., Wiesner, M. R., and Nel, A. E. (2006). Comparison of the abilities of ambient and manufactured nanoparticles to induce cellular toxicity according to an oxidative stress paradigm. *Nano Lett.* **6,** 1794-1807.

Xiao, G. G., Wang, M., Li, N., Loo, J. A., and Nel, A. E. (2003). Use of proteomics to demonstrate a hierarchical oxidative stress response to diesel exhaust particle chemicals in a macrophage cell line. *J. Biol. Chem.* **278,** 50781-50790.

Yanagisawa, R., Takano, H., Inoue, K. I., Ichinose, T., Sadakane, K., Yoshino, S., Yamaki, K., Yoshikawa, T., and Hayakawa, K. (2006). Components of diesel exhaust particles differentially affect Th1/Th2 response in a murine model of allergic airway inflammation. *Clin. Exp. Allergy* **36,** 386-395.

Yang, H. M., Antonini, J. M., Barger, M. W., Butterworth, L., Roberts, B. R., Ma, J. K., Castranova, V., and Ma, J. Y. (2001). Diesel exhaust particles suppress macrophage function and slow the pulmonary clearance of Listeria monocytogenes in rats. *Environ. Health Perspect.* **109,** 515-521.

Yin, X. J., Dong, C. C., Ma, J. Y., Roberts, J. R., Antonini, J. M., and Ma, J. K. (2007). Suppression of phagocytic and bactericidal functions of rat alveolar macrophages by the organic component of diesel exhaust particles. *J .Toxicol. Environ. Health A* **70,** 820-828.

Yin, X. J., Schafer, R., Ma, J. Y., Antonini, J. M., Weissman, D. D., Siegel, P. D., Barger, M. W., Roberts, J. R., and Ma, J. K. (2002). Alteration of pulmonary immunity to Listeria monocytogenes by diesel exhaust particles (DEPs). I. Effects of DEPs on early pulmonary responses. *Environ. Health Perspect.* **110,** 1105-1111.

Zhang, P., Summer, W. R., Bagby, G. J., and Nelson, S. (2000). Innate immunity and pulmonary host defense. *Immunol. Rev.* **173,** 39-51.

Zheng, M., Cass, G. R., Schauer, J. J., and Edgerton, E. S. (2002). Source apportionment of PM2.5 in the Southeastern United States using solvent-extractable organic compounds as tracers. *Environ. Sci. Technol.* **36,** 2361-2371.

Zinkernagel, R. M. (2000). Localization dose and time of antigens determine immune reactivity. *Semin. Immunol.* **12,** 163-171.